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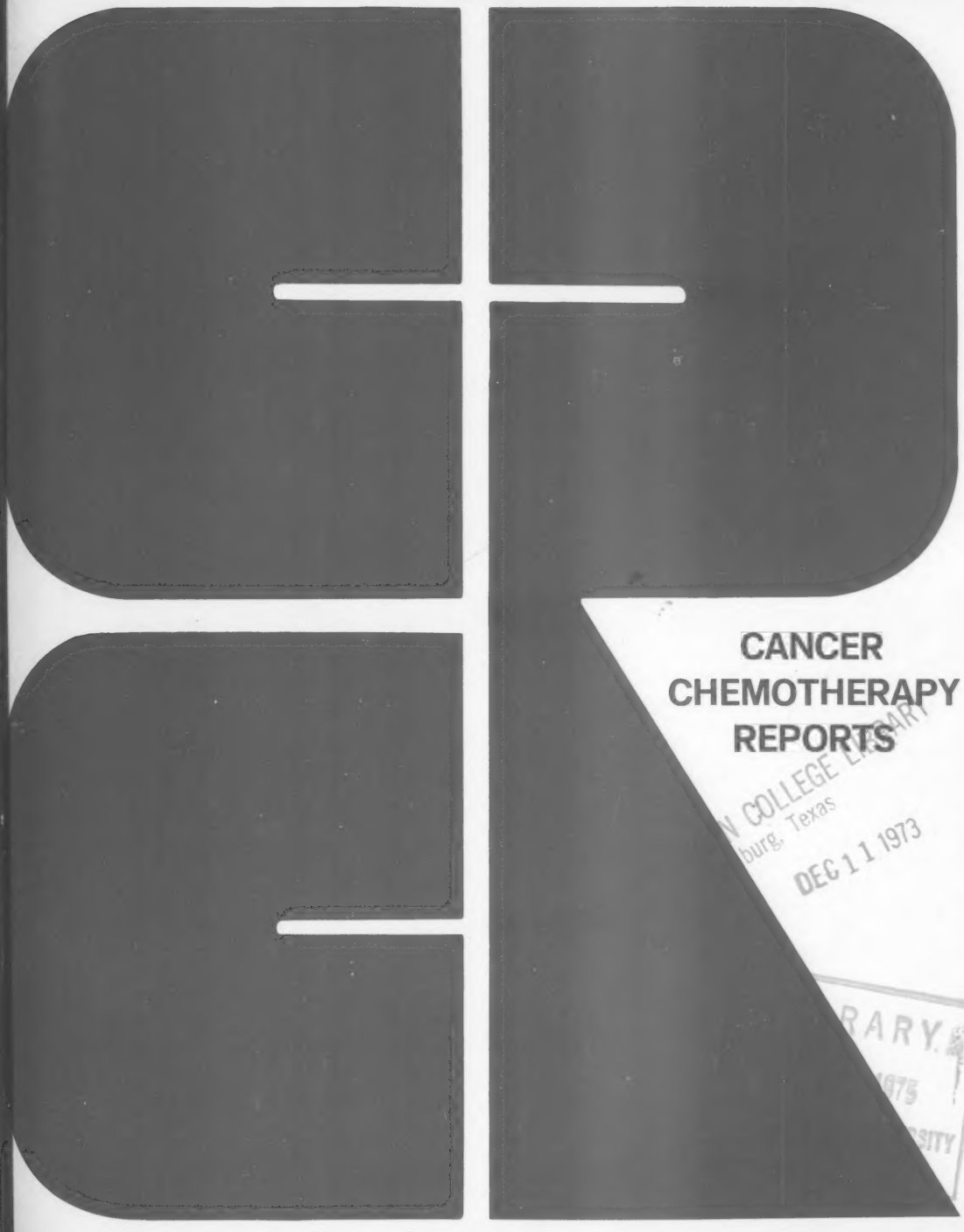

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Contents

Cancer Chemotherapy Reports
Vol. 57, No. 3
September/October 1973

ARTICLES

Page

Chemotherapy of Morris Hepatoma 3924A: Correlation of Size and Weight of Tumor and Preliminary Data With 5-Fluoro-2'-deoxyuridine (5-FUDR; NSC-27640) 245

K. W. Lo, E. E. Miller, H. P. Morris, and K. C. Tsou
University of Pennsylvania, Philadelphia

Structure-Activity Correlations of Anticancer Agents: Diaminopyrimidines, *N*-Acyltriamines, Bis-(1-aziridinyl)-phosphinyl Carbamates, and Aromatic Nitrogen Mustards 251

Eric J. Lien and George L. Tong
University of Southern California, Los Angeles

Evaluation of 4-Carbethoxy-5-(3,3-dimethyl-1-triazeno)-2-phenylimidazole (NSC-166721) and 4-Carbethoxy-5-(3,3-dimethyl-1-triazeno)-2-methylimidazole (NSC-166722) as Potential Antitumor Agents 263

James Heyes, Elaine J. Catherall, and Andrew Cockburn
Beecham Research Laboratories, Betchworth, Surrey, England

Cytotoxic, Antiviral, and Antitumor Activity of Some Derivatives of Daunomycin (NSC-82151) 269

A. Di Marco, A. M. Casazza, T. Dasdia, F. Giuliani, L. Lenaz, A. Necco, and C. Soranzo
Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy

Disposition of Arabinosylcytosine (NSC-63878) and 6-Thioguanine (NSC-752) in Solid L1210 Leukemia Tumor-Bearing Mice 275

Robert F. Pittillo and Carolyn Woolley
Southern Research Institute, Birmingham, Alabama

Value of Cytosine Arabinoside (NSC-63878) Plus 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) Therapy in Advanced Murine L1210 Leukemia and Enhancement of the Combination With Sequential Doses of Methotrexate (NSC-740) or 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU; NSC-409962) 291

I. Kline, R. J. Woodman, M. Gang, R. L. Cysyk, and J. M. Venditti
Microbiological Associates, Bethesda, Maryland

Protection With *N*-Acetyl-L-cysteine (NSC-111180) Against Isophosphamide (NSC-109724) Toxicity and Enhancement of Therapeutic Effect in Early Murine L1210 Leukemia 299

I. Kline, M. Gang, R. J. Woodman, R. L. Cysyk, and J. M. Venditti
Microbiological Associates, Bethesda, Maryland

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Page

Effect of Streptonigrin (NSC-45383) and Analogs on Oncornavirus Replication and DNA Polymerase Activity 305

Michael A. Chirigos, John W. Pearson, Takis S. Papas, Wilna A. Woods, Harry B. Wood, Jr., and Gerald Spahn
National Cancer Institute, Bethesda, Maryland

Preliminary Clinical Trials of Butocin (NSC-172755) in Patients With Generalized Carcinoma of the Breast 311

O. Andrysek, Z. Dienstbier, M. Semonský, and J. Zámecník
Charles University, Prague, Czechoslovakia

Treatment of Acute Leukemia With 5-Azacytidine (NSC-102816) 319

Kenneth B. McCredie, Gerald P. Bodey, Michael A. Burgess, Jordan U. Gutterman, Victorio Rodriguez, Margaret P. Sullivan, and Emil J Freireich
The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston

Phase II Clinical Trials of BCNU (NSC-409962) and Bleomycin (NSC-125066) in the Treatment of Kaposi's Sarcoma 325

Charles L. Vogel, Dennis Clements, Alex K. Wanume, Thomas Toya, Aron Primack, and Sebastian Kyalwazi
Uganda Cancer Institute and Makerere University Medical School, Kampala

5-Fluorouracil (NSC-19893) and Actinomycin D (NSC-3053) in the Treatment of Acute Leukemia in Children 335

Vita J. Land, Paul G. Dyment, Diane Komp, G. Bennett Humphrey, Margaret P. Sullivan, and Kenneth A. Starling
Southwest Cancer Chemotherapy Study Group

Phase I Study of Chromomycin A_3 (NSC-58514) 341

J. S. Kovach, C. G. Moertel, D. L. Ahmann, R. G. Hahn, A. J. Schutt, and J. V. Donadio, Jr.
Columbia University, New York, New York

BRIEF REPORTS AND PRELIMINARY COMMUNICATIONS 349

Studies on Drug Resistance. V. Combination Chemotherapy With Cytosine Arabinoside (NSC-63878) Plus 6-Thioguanine (NSC-752) in Cytosine Arabinoside-Sensitive and -Resistant P815 Mouse Neoplasms

W. Kreis
Memorial Sloan-Kettering Cancer Center, New York, New York

Treatment In Vivo of R3230AC Carcinoma of the Rat With Estradiol Mustard (NSC-112259) or its Molecular Components 353

Richard B. Everson, Thomas C. Hall, and James L. Wittliff
University of Rochester, New York

Page

Phase II Study of *Cis*-diamminedichloroplatinum (NSC-119875) in Advanced Carcinoma of the Large Bowel 357

J. S. Kovach, C. G. Moertel, A. J. Schutt, R. G. Reitemeier, and R. G. Hahn
Columbia University, New York, New York

High-Dose Intermittent Intravenous Infusion of Procarbazine (NSC-77213) 361

B. A. Chabner, R. Sponzo, S. Hubbard, G. P. Canellos, R. C. Young, P. S. Schein, and V. T. DeVita
National Cancer Institute, Bethesda, Maryland

Antitumor Activity of a Homo-aza-steroidal Ester of [p-[Bis(2-chloroethyl)-amino]phenyl]acetic Acid (NSC-71964) 365

P. Catsoulacos and L. Boutis
Theagenion Cancer Institute, Thessaloniki, Greece

ANNOUNCEMENTS

American College of Physicians: Course on Advances in Childhood Cancer 369

Traineeships in Clinical Oncology 369

Annual Meeting of the American Association for Cancer Research 370

Chemotherapy of Morris Hepatoma 3924A: Correlation of Size and Weight of Tumor and Preliminary Data With 5-Fluoro-2′-deoxyuridine (5-FUDR; NSC-27640) [1,2,3]

K. W. Lo,[4] E. E. Miller,[4] H. P. Morris,[5] and K. C. Tsou [4,6]

SUMMARY

The volumes calculated from external measurements of the tumors in situ bear a linear relationship to the wet weights of the Morris hepatoma 3924A. Treatment of this rat tumor with 5-fluoro-2′-deoxyuridine at 30 mg/kg for 7 days showed an initial significant reduction with subsequent regrowth of the tumor to the control size.

[Cancer Chemother Rep 57:245–249, 1973]

In the evaluation of the effectiveness of chemotherapeutic agents against solid tumors, it is desirable to have a convenient and reliable in situ method of monitoring the growth or regression of tumors after treatment. While quantitative measurements of tumor growth have been studied extensively, only recently has attention been paid to the correlation of the tumor size with its weight. For instance, Dethlefsen, Prewitt, and Mendelsohn (1), during their detailed study of tumor growth curves, showed the linear relationship between tumor volume and weight in mammary tumors of C3H mice. In conjunction with our chemotherapy studies of hepatomas, we have chosen Morris hepatoma 3924A as a useful animal model, and have undertaken a study of the size and weight relationship of this tumor. Such correlation is a necessary prerequisite, since in practice only tumor size can be determined during treatment and throughout the tumor growth period. With this data, one can then deduce the tumor weight and provide more meaningful correlation of the drug effect when the host weight also varies.

MATERIALS AND METHODS

Tumors

Morris hepatoma 3924A was transplanted subcutaneously (sc) to both sides of ACI rats. A random sampling of 15 tumors was used in the size and weight correlation study. Animals were killed on Day 32 after tumor transplantation.

5-FUDR Treatment

Treatment with 5-FUDR began on the 7th day after tumor transplantation, with 30 mg/kg of 5-FUDR in 0.85% sodium chloride given intraperitoneally daily for 7 days. The control rats were injected with sodium chloride simultaneously. Six rats were used in each group.

Measurement of Tumor Mass and Size

Tumor mass was determined by weighing the freshly extirpated tumors. Measurement of tumor size was made with vernier calipers in three dimensions, length, width, and height,

[1] Received May 11, 1972; revised June 11, 1973; accepted June 21, 1973.

[2] Supported by Public Health Service grant CA-07339 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] 5-FUDR was supplied by Drug Research and Development, Division of Cancer Treatment, NCI.

[4] Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania, Philadelphia.

[5] Department of Biochemistry, College of Medicine, Howard University, Washington, DC.

[6] *Reprint requests to:* K.C. Tsou, PhD, Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania, Philadelphia, Pa 19104.

referred to as d_1, d_2, and d_3 respectively. Hair was shaved from the area of tumor growth to facilitate measurement.

Calculation of Tumor Size

Although the hepatomas had a wide range of sizes, they were all approximated to ellipsoids or spheres in shape. Volumes of tumors were therefore calculated on both of these geometrical configurations.

(*a*) Ellipsoid:

$$V = \frac{4\pi}{3}\left(\frac{d_1}{2}\right)\left(\frac{d_2}{2}\right)\left(\frac{d_3}{2}\right) = \frac{\pi}{6} d_1 d_2 d_3 \quad (1)$$

where V is the volume of the tumor and is related to the mass (M) of the tumor by the following equation:

$$V = \frac{M}{\rho} \quad (2)$$

where ρ is the specific gravity of the tumor. The following equation is derived from equations (1) and (2):

$$d_1 d_2 d_3 = \frac{6}{\rho\pi} \cdot M \quad (3)$$

Hence, a plot of the product $d_1d_2d_3$ versus M should give a straight line from the slope of which the density of the tumor can be calculated.

(*b*) Sphere: Here we define $\bar{d}$ as the average of d_1,d_2,d_3, ie,

$$\bar{d} = \frac{d_1 + d_2 + d_3}{3} \quad (4)$$

$$V = \frac{4\pi}{3}\left(\frac{\bar{d}}{2}\right)^3 \quad (5)$$

and

$$(\bar{d})^3 = \frac{6}{\rho\pi} \cdot M \quad (6)$$

Equation (6) is mathematically equivalent to equation (3). The specific gravity of the tumor can therefore be obtained as above.

Calculation of Host Weight

The host weight of rats used in chemotherapy was calculated by subtracting the weight of tumors from the total weight of the individual rat. The weights of the tumors were in turn obtained from the size measurements of d_1,d_2,d_3 and the slope of figure 1 or 2.

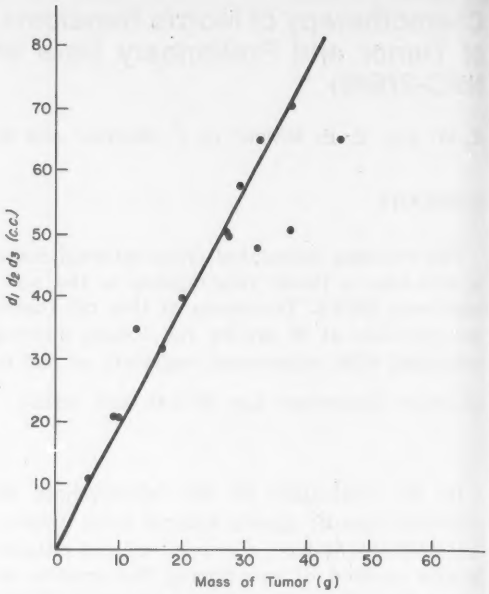

FIGURE 1.—Plot of d_1 d_2 d_3 against tumor mass.

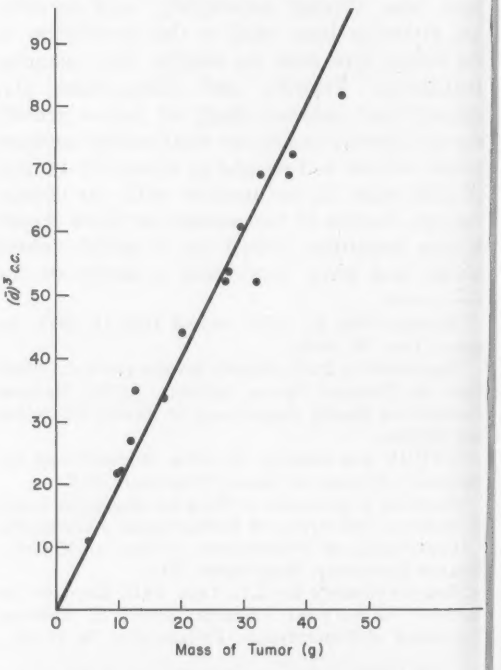

FIGURE 2.—Plot of $(\bar{d})^3$ against tumor mass.

Specific Gravity of Tumor

The specific gravity (density) of the tumor was found by means of Archimede's principle. The tumor was tied to a piece of thread and hung from the hook of a single pan Mettler balance (type B6). Its mass was determined. A cardboard box of suitable dimensions was cut into the form of a bridge and placed across the pan of the balance so that on releasing the pan, it made no contact with the pan. The tumor was then immersed in a beaker of physiologic saline supported by the bridge (fig 3). The apparent mass of the tumor was determined. The difference between these two masses represents the buoyancy of the saline on the tumor, which by Archimede's principle is equal to the volume of the saline displaced by the tumor.

$$\therefore \rho = \frac{\text{mass of tumor and thread in air} - \text{mass of thread in air}}{\text{mass of tumor in air} - \text{mass of tumor in water}} \times \text{specific gravity of saline}$$

The specific gravity of physiologic saline is extrapolated to be 1.0066 (2).

RESULTS AND DISCUSSION

Size and Weight Correlation of Tumors

A straight line was obtained from a plot of $d_1\ d_2\ d_3$ versus tumor mass (fig 1). From the slope of this line, the specific gravity of the tumor was calculated to be 0.98. An essentially identical line was obtained when $\bar{d}^3$ was plotted versus tumor mass (fig 2). The specific gravity was calculated to be 0.96 in this case. These results suggest that we can use either the prolate ellipsoid or the average sphere to calculate the volume of this tumor. The few points that do not fall on the line in both cases may represent tumors of rather odd shapes. Comparing figures 1 and 2, it is interesting to note that this tumor is closer in shape to a sphere than an ellipsoid. Both of our specific gravity values are about 5% lower than that determined directly from Archimede's principle, which was found to be 1.030. This may be due to the fact that we have ignored the skin thickness in our estimation of tumor size. Nonetheless, the in situ measurements of the dimensions of the tumor correlate exceedingly well with the wet weights of the tumors. Consequently, it is not necessary to kill the animal in order to determine the weight of the tumor during treatment with chemotherapeutic agents. This leads to a savings not only in animals, but also in time and energy of the experimenter. The toxicity of a drug could affect the weight of the host, and by using the size and weight correlation the tumor weight can be deducted from the total weight to provide such data. Although our study is confined to only Morris hepatoma 3924A, this method can be extended to all other sc implanted tumors. Our method of determining the specific gravity represents a more convenient and accurate way of finding this physical parameter than that of suspending the tumor in a series of solutions of $CuCl_2$ of known specific gravity (1).

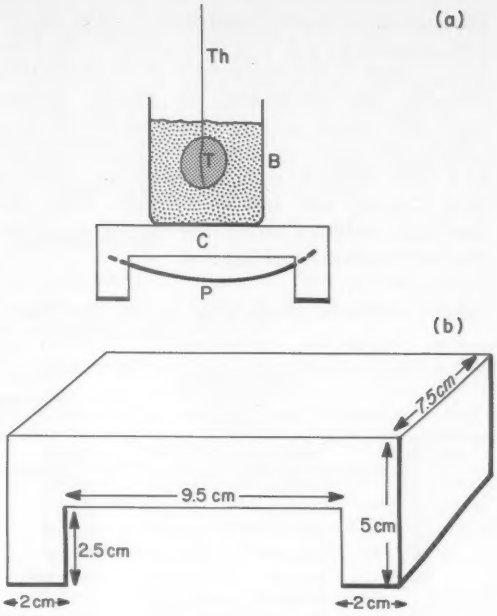

FIGURE 3.—(*a*) Schematic diagram for the determination of the specific gravity of tumor. H = hook of balance; Th = thread; T = tumor; B = beaker containing physiologic saline; C = cardboard; and P = balance pan. (*b*) Dimensions of cardboard bridge used.

Effect of 5-FUDR on Growth of Morris Hepatoma 3924A

In spite of the fact that 5-FUDR has been

used clinically in the treatment of colon cancer metastasized to the liver and occasionally hepatoma, both in the University of Pennsylvania Hospital and elsewhere, no animal studies have been noted. Morris hepatoma 3924A is a poorly differentiated tumor and therefore may be a close model to colon-metastasized cases in man that we can use at present. With the treatment outlined above, the results are summarized in table 1 and figure 4.

As can be seen in table 1, because of the statistical variation only significant differences ($P<0.05$) should be considered. In spite of the inherent difficulties in this type of work, the following results are apparent.

5-FUDR is effective in reducing the size of Morris hepatoma 3924A on Days 11–35. This effect is relatively fast but does not last long, probably until 7 days after the cessation of injection.

TABLE 1.—Changes (mean ± SE) in total rat weight, host weight, and tumor size during and after 5-FUDR chemotherapy of Morris hepatoma 3924A

Days after transplant	Total weight of rat (g)		Host weight* (g)		$\overline{d_1\ d_2\ d_3}$ (cc)		
	NaCl	5-FUDR	NaCl	5-FUDR	NaCl	5-FUDR	Significant level†
6	171±9	175±5	171±9	175±5	0.23±0.04	0.23±0.09	$P > 0.05$
11	177±9	175±4	176±9	174±4	1.03±0.69	0.39±0.14	$P < 0.01$
23	207±7	209±7	200±9	206±8	7.39±4.23	3.22±1.22	$0.01 < P < 0.05$
28	228±6	223±5	203±9	211±8	23.2±12.5	10.9±5.3	$0.01 < P < 0.05$
32	254±9	248±12	212±10	221±11	39.5±18.3	25.2±11.3	$0.01 < P < 0.05$
35	279±13	273±15	217±12	231±9	57.5±27.7	38.0±18.2	$P > 0.05$
39	310±35	292±23	226±18	216±19	77.3±35.5	70.1±33.1	$P > 0.05$
43	329±30	322±35	190±20	193±28	128±57	120±45	$P > 0.05$
47	375±43	367±32	205±10	186±9	158±63	167±50	$P > 0.05$
50	393±42	373±21	172±25	184±23	201±69	176±50	$P > 0.05$

*Host weight = rat weight − tumor weight.
†Analyzed by the Mann-Whitney "u" test.

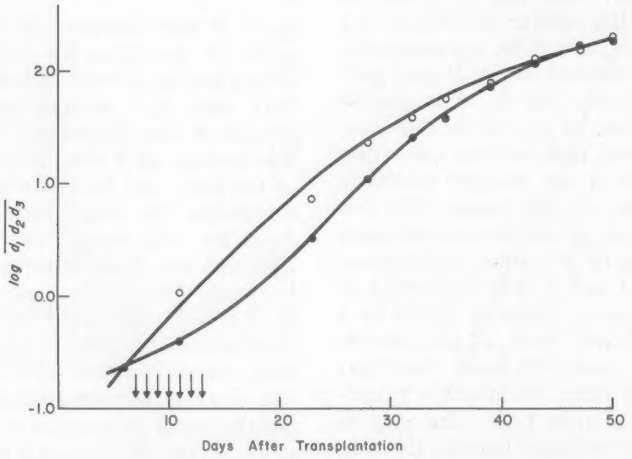

FIGURE 4.—Change in tumor size during and after 5-FUDR chemotherapy of Morris hepatoma 3924A. $\overline{d_1\ d_2\ d_3}$ = mean of $d_1\ d_2\ d_3$. ○ = NaCl control; ● = 5-FUDR-treated. ▼ = days when drugs were given.

Taking into account the weight as derived by our method, the increase in total rat weight in the period from 23 to 50 days is primarily in the tumor, as the host weights are fairly constant from 23 to 47 days (table 1). Furthermore, the excellent agreement between the host weights of the 5-FUDR-treated and the control groups may reflect the low toxicity of 5-FUDR to the host, which is why it is a useful palliative drug for clinical purposes.

It is also apparent that, in spite of the initial successful onslaught of this drug to the tumor, the tumor growth was resumed at a faster rate and eventually resulted in no difference in the survival times of treated (53 ± 5 days) and control (53 ± 5 days) groups. This type of drug resistance has been attributed in the past to the decrease in thymidine kinase in the resistant tumor cell population, therefore in vivo synthesis of the active drug 5-FUDR monophosphate [†] decreases (3). It has been suggested by us that if this were the responsible mechanism for drug resistance, the phosphodiester of 5-FUDR (4) should be used as an alternate drug when such resistance developed. Further work is necessary to better understand the resistance problem of 5-FUDR.

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[†] 5-FUDR monophosphate: NSC-83097; uridine, 2'-deoxy-5-fluoro-, 5'-phosphate.

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Structure-Activity Correlations of Anticancer Agents: Diaminopyrimidines, *N*-Acyltriamines, Bis-(1-aziridinyl)-phosphinyl Carbamates, and Aromatic Nitrogen Mustards [1,2]

Eric J. Lien and George L. Tong [3,4]

SUMMARY

The anticancer activities of a series of dihydrofolate reductase inhibitory diaminopyrimidines, solapalmitine-related *N*-acyltriamines, alkylating agents like bis-(1-aziridinyl)-phosphinyl carbamates, and various aromatic nitrogen mustards are correlated with substituent constants like E_s, π, log P, σ, and σ^*. Good to fair correlations are obtained for the activity of these compounds against various types of cancer or cancer cells as well as for their toxicity.

[Cancer Chemother Rep 57:251–261, 1973]

Considerable progress has been made by Hansch and his co-workers in quantitative structure-activity relationships in the area of antineoplastic agents (1,2). Although many thousands of alkylating agents and antimetabolites have been synthesized and tested for antitumor activity (3–5), relatively few sets of quantitative data are available for regression analysis using well-defined physicochemical constants. The purpose of this report is to extend this approach to as many of these sets of data as are available in order to reveal some possible guidelines for further drug design.

METHODS

All of the equations listed in table 1 have been derived via the method of nonweighted least squares fit using an IBM 370/155 computer.

For this type of quantitative structure-activity relationship, the biologic activity from the literature (6–8) is expressed as the log $1/C$, where C is the concentration of the drug in moles per kilogram required to cause a standard biologic response, eg, ED50, LD50, ID50.

The physicochemical constants used in the regression analysis are either taken from the literature (9–13) or calculated (*see* table 2). They can be described briefly as follows:

Log P is the log of the partition coefficient of a compound between *n*-octanol and water.

π is the log of the octanol-water partition coefficient for a substituent. Taking advantage of the additive and constitutive nature of log P, one can get log $P = \Sigma\pi$.

σ Hammett's sigma constant is a measure of the electronic effect on an aromatic ring when a substituent is attached.

σ^* Taft's polar substituent constant is a measure of the electronic effect of a substituent in an aliphatic system.

E_s Taft's steric parameter is a measure of intramolecular steric effects.

(For further information on substituent constants *see* references 13 and 14.)

[1] Received Dec 11, 1972; revised Apr 2, 1973; accepted May 9, 1973.

[2] Supported in part by general research support grant RR-05702-03 from the National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

[3] School of Pharmacy, University of Southern California, Los Angeles.

[4] *Address correspondence to:* Eric J. Lien, PhD, School of Pharmacy, University of Southern California, University Park, Los Angeles, Calif 90007.

A few examples for the calculation of log P and π values are given as the following:

(*a*) The π value of the adamantyl group is (2,9):

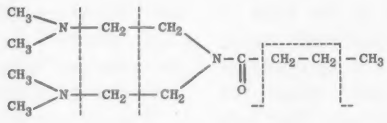
$$\pi_{\text{(adamantyl)}} = \log P_{\text{(1-hydroxyadamantane)}} - \pi_{\text{(OH)}}$$
$$= 2.14 - (-1.16)$$
$$= 3.30.$$

Therefore:

$$\log P_{\substack{\text{(5-adamantyl-6-methyl-2,}\\ \text{4-diaminopyrimidine)}}} = \pi_{\text{(2-aminopyrimidine)}} + \pi_{(NH_2)} + \pi_{\text{(adamantyl)}} + \pi_{(CH_3)}$$
$$= -0.22 + 0.18 + 3.30 + 0.50$$
$$= 3.76.$$

Since the E_s value of the adamantyl group is not available, the E_s value of $-C(C_2H_5)_3$ is used for the first approximation.

(*b*) The log P values for the N-acyltriamines are calculated as follows:



$$\log P_{\text{(calculated)}} = 2\left[\pi_{N(CH_3)_2} + \pi_{(CH_2)}\right] + \log P_{(N,N\text{-dimethylacetamide})} + 2\left[\pi_{(CH_2)}\right]$$
$$= 2[-0.30 + 0.50] + (-0.77) + 2[0.50]$$
$$= 0.40 + (-0.77) + 1.00$$
$$= 0.63.$$

For the π_{acyl} values, 0.5 is used for each CH_2 unit or CH_3. Therefore, for the above compound, $\pi_{acyl} = 3[0.50] = 1.50$.

TABLE 1.—Equations correlating various physicochemical constants with anticancer activity or toxicity

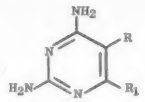
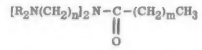
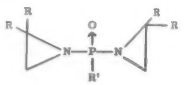
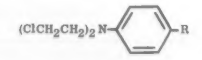
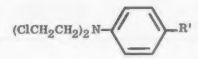
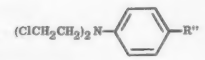
Compound, structure	Test system	Equation	n*	r†	s‡	Equation No.
Diaminopyrimidines 	Mouse TA 3 carcinoma cells in vitro	$\log 1/ID_{50} = 0.378 \log P + 4.604$	9	0.359	1.048	1
		$\log 1/ID_{50} = -0.762\ E_{s(R)} + 5.380$	9	0.822	0.639	2
		$\log 1/ID_{50} = -0.763\ E_{s(R)} + 0.381 \log P + 3.985$	9	0.898	0.533	3
		$\log 1/ID_{50} = -0.679\ E_{s(R)} - 0.260 (\log P)^2 + 2.306 \log P + 0.712$ ($\log P_o = 4.44 \pm \infty$)	9	0.946	0.431	4
N-Acyltriamines 	Cell culture of human carcinoma of the nasopharynx (KB assay)	$\log 1/ED_{50} = -0.043 (\log P)^2 + 0.794 \log P + 2.515$ $\log P_o = 9.17$; (7.85—14.17)	28	0.784	0.660	5
		$\log 1/ED_{50} = -0.093(\pi_{acyl})^2 + 1.340\ \pi_{acyl} + 1.400$ $\pi_{o(acyl)} = 7.24$; (6.59—8.45)	28	0.849	0.563	6
		When $n = 4$, $\log 1/ED_{50} = -0.105 (\log P)^2 + 1.797 \log P - 1.560$ $\log P_o = 8.56$; (8.16—9.14)	13	0.943	0.278	7
Bis-(1-aziridinyl)-phosphinyl carbamates 	Walker 256 carcinoma in rats	$\log 1/ED_{50} = 0.059\sigma^*_{(R')} + 3.903$	10	0.065	0.792	8
		$\log 1/ED_{50} = -0.379\ \Sigma\pi + 4.706$	10	0.712	0.557	9
		$\log 1/ED_{50} = 2.739\sigma^*_{(R)} + 3.344$	10	0.945	0.259	10
	Toxicity in rats	$\log 1/LD_{50} = 0.092\sigma^*_{(R')} + 3.281$	10	0.130	0.008	11
		$\log 1/LD_{50} = -0.157\ \Sigma\pi + 3.740$	10	0.381	0.567	12
		$\log 1/LD_{50} = 1.727\sigma^*_{(R)} + 3.032$	10	0.771	0.391	13
Aromatic nitrogen mustards 	Walker 256 carcinoma in rats	$\log 1/ED_{50} = -0.263\pi_{(R)} - 1.623\sigma_{(R)} + 3.660$	9	0.839	0.448	14
R = miscellaneous	Toxicity in rats	$\log 1/LD_{50} = -0.269\pi_{(R)} - 1.577\sigma_{(R)} + 3.476$	9	0.927	0.273	15

TABLE 1.—Equations correlating various physicochemical constants with anticancer activity or toxicity—Continued

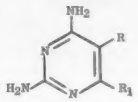
Compound, structure	Test system	Equation	n*	r†	s‡	Equation No.
Aromatic nitrogen mustards	Toxicity in rats	$\log 1/LD_{10} = -1.725\sigma_{(R')} + 3.681$	7	0.907	0.245	16
						
$R' = -N-R''$						
	Walker 256 carcinoma in rats	$\log 1/ED_{10} = -0.286\pi_{(R'')} + 4.618$	6	0.510	0.594	17
$R'' = -O-R'$ or $-Ph-O-R$		$\log 1/ED_{10} = -0.648(\pi)^2_{R''} + 0.441\pi_{(R'')} + 5.070$ ($\pi_o = 0.34$; 0.07—0.50)	6	0.980	0.160	18
	Toxicity in rats	$\log 1/LD_{10} = -0.373\pi_{R''} + 4.021$	7	0.904	0.241	19

*No. of compounds tested in each series.
†Correlation coefficient of the regression.
‡Standard deviation.

TABLE 2.—Biologic data and the physicochemical constants used in the stepwise multiple regression analysis

Compound: Diaminopyrimidines

Structure:

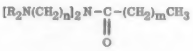


Test system: Mouse TA 3 carcinoma cells in vitro

		log 1/ID_{50}			
$E_{s(R)}$	log *P*	Observed [a]	Calculated [b]	*R*	R_1
−0.36	1.96	4.28	4.48	*n*-C_3H_7	CH_3
−0.40	2.96	5.52	5.54	*n*-C_5H_{11}	CH_3
−0.40 [c]	3.46	5.60	5.86	*n*-C_6H_{13}	CH_3
−0.40 [c]	3.96	5.80	6.05	*n*-C_7H_{15}	CH_3
−0.33	4.46	5.64	6.06	*n*-C_8H_{15}	CH_3
−0.33 [c]	5.46	5.96	5.79	*n*-$C_{10}H_{21}$	CH_3
−0.76	2.97	6.40	5.79	*c*-C_6H_{11}	CH_3
−3.80 [c]	3.76	8.22	8.29	$C_{10}H_{15}$ (adamantyl)	CH_3
−0.40 [c]	3.97	6.47	6.05	*n*-C_6H_{13}	C_2H_5

Compound: *N*-acyltriamines

Structure:



Test system: Human carcinoma of the nasopharynx cell culture (KB assay)

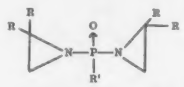
log 1/ED_{50}

π_{acyl}	log *P*	*n*	*m*	*R*	Observed [d]	Calculated [e]	Calculated [f]	Calculated [g]
1.50	0.63	2	2	CH_3	3.41	3.00	3.20	—
3.50	2.63	2	6	CH_3	4.11	4.31	4.96	—
5.50	4.63	2	10	CH_3	5.87	5.26	5.97	—
7.50	6.63	2	14	CH_3	6.08	5.88	6.24	—
1.50	1.63	3	2	CH_3	3.91	3.70	3.20	—
3.50	3.63	3	6	CH_3	5.08	4.83	4.96	—
5.50	5.63	3	10	CH_3	6.95	5.61	5.97	—
7.50	7.63	3	14	CH_3	7.25	6.05	6.24	—
2.50	3.63	4	4	CH_3	3.73	4.83	4.17	3.58
3.50	4.63	4	6	CH_3	4.15	5.26	4.96	4.51
4.50	5.63	4	8	CH_3	5.17	5.61	5.56	5.23
5.50	6.63	4	10	CH_3	6.15	5.88	5.97	5.74
6.50	7.63	4	12	CH_3	6.07	6.05	6.20	6.04
7.50	8.63	4	14	CH_3	6.10	6.14	6.24	6.13
8.50	9.63	4	16	CH_3	6.24	6.15	6.10	6.01
9.50	10.63	4	18	CH_3	5.50	6.06	5.78	5.68
10.50	11.63	4	20	CH_3	5.43	5.89	5.26	5.14
3.50	5.63	5	6	CH_3	4.61	5.61	4.96	—
5.50	7.63	5	10	CH_3	5.77	6.05	5.97	—
7.50	9.63	5	14	CH_3	5.72	6.15	6.24	—
5.50	8.63	6	10	CH_3	6.21	6.14	5.97	—
7.50	10.63	6	14	CH_3	6.22	6.06	6.24	—
5.50	9.63	7	10	CH_3	7.36	6.15	5.97	—
7.50	11.63	7	14	CH_3	6.41	5.89	6.24	—
5.50	7.63	4	10	C_2H_5	5.88	6.05	5.97	6.04
7.50	9.63	4	14	C_2H_5	5.81	6.15	6.24	6.01
5.50	8.23	4	10	*i*-C_3H_7	6.37	6.12	5.97	6.12
7.50	10.23	4	14	*i*-C_3H_7	5.45	6.11	6.24	5.83

TABLE 2.—Biologic data and the physicochemical constants used in the stepwise multiple regression analysis—Continued

Compound: Bis-(1-aziridinyl)-phosphinyl carbamates

Structure:

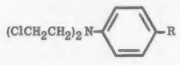


Test system: Walker 256 carcinoma in rats

σ^*_R	$\Sigma\pi_{(4R+R')}$	$\sigma^*_{R'}$	log 1/ED_{50} Observed [h]	log 1/ED_{50} Calculated [i]	log 1/LD_{50} Observed [h]	log 1/LD_{50} Calculated [j]	R	R'
0.49	−0.74	1.99	4.80	4.69	3.70	3.88	H	$NHCOOEt$
0.49	0.89	1.99 [e]	4.60	4.69	3.60	3.88	H	$NHCOOCH_2Ph$
0.49	1.00	1.99 [e]	4.46	4.69	3.66	3.88	H	$NHCOOCH_2Ph$-(p-NO_2)
0.49	1.13	1.99 [e]	4.68	4.69	3.66	3.88	H	$NHCOOCH_2Ph$-(m-NO_2)
0.00	1.26	1.99 [e]	3.29	3.34	2.82	3.03	CH_3	$NHCOOEt$
0.00	2.89	1.99 [e]	2.88	3.34	2.77	3.03	CH_3	$NHCOOCH_2Ph$
0.00	2.48	−0.35	3.79	3.34	3.34	3.03	CH_3	OEt
0.00	3.61	2.43 [e]	3.42	3.34	3.12	3.03	CH_3	OPh
0.00	3.85	2.43 [e]	3.34	3.34	3.11	3.03	CH_3	$OPh(p\text{-}NO_2)$
0.49	1.85	2.43 [e]	4.89	4.69	4.77	3.88	H	$OPh(p\text{-}NO_2)$

Compound: Aromatic nitrogen mustards

Structure:

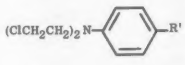


Test system: Walker 256 carcinoma in rats

π_R	σ_R	log 1/ED_{50} Observed [k]	log 1/ED_{50} Calculated [l]	log 1/LD_{50} Observed [k]	log 1/LD_{50} Calculated [m]	R
0.00	0.00 [n]	3.40	3.66	3.44	3.48	H
−0.28	0.27 [n]	3.30	3.30	3.04	3.13	COOH
−1.82	0.62 [n]	2.82	3.13	2.95	2.99	SO_2NH_2
−0.67	−0.36 [n]	4.49	4.42	4.13	4.22	OH
−1.23	−0.66 [n]	4.70	5.05	4.82	4.85	NH_2
−0.97	−0.02 [n]	4.47	3.95	3.99	3.77	$NHCOCH_3$
−2.16	−0.02 [e]	4.47	4.26	4.47	4.09	$NHCOCH_2NH_2$
−1.94	−0.02 [e]	4.80	4.20	4.17	4.03	$NHCOCH_2NHCOCH_3$
−2.43	−0.02 [e]	3.85	4.33	3.70	4.16	$NHCOCH_2NHCOOCH_3$

Compound: Aromatic nitrogen mustards

Structure:



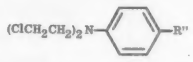
Test system: Toxicity in rats

$\pi_{R'}$	$\sigma_{R'}$	log 1/LD_{50} Observed [o]	log 1/LD_{50} Calculated [p]	R'
−1.23	−0.66 [n]	4.82	4.82	$NH_2 \cdot HCl$
−0.44	0.08 [e]	3.27	3.54	$NHCOOCH(CH_3)_2$
0.39	0.08 [e]	3.25	3.54	$NHCOOPh$
0.11	0.08 [e]	3.84	3.54	$NHCOOPh(4\text{-}COOH)$
0.11	0.08 [e]	3.77	3.54	$NHCOOPh(3\text{-}COOH)$
0.38	0.08 [e]	3.57	3.54	$NHCOOPh(4\text{-}COOCH_3)$
0.38	0.08 [e]	3.56	3.54	$NHCOOPh(2\text{-}COOCH_3)$

TABLE 2.—Biologic data and the physicochemical constants used in the stepwise multiple regression analysis—Continued

Compound: Aromatic nitrogen mustards

Structure:



Test system: Walker 256 carcinoma in rats

		log 1/ED_{50}		log 1/LD_{50}		
π_R''	σ_R''	Observed [q]	Calculated [r]	Observed [q]	Calculated [s]	R''
−0.67	−0.36 [n]	4.46	4.48	4.09	4.27	OH(HCl)
−0.64	0.31 [t]	4.58	4.52	4.26	4.26	$OCOCH_3$
0.99	0.31 [o]	4.82	4.87	4.03	3.65	OCOPh
1.99	0.31 [e]	3.27	3.38	3.07	3.28	OCOPh(2,6(CH_3)$_2$)
1.49	0.31 [e]	4.51	4.29	3.68	3.47	OCOPh(2–CH_3)
0.40	0.31 [e]	5.05	5.14	3.82	3.87	OCONHPh(4–COOH)
2.53	0.01 [e]	—	—	2.93	3.08	PhOCONHPh(4–COOH)

a. Observed value from reference 6.
b. Calculated from equation 4.
c. Estimated value.
d. Observed value from reference 7.
e. Calculated from equation 5.
f. Calculated from equation 6.
g. Calculated from equation 7.
h. Observed value from table 8 of reference 8.
i. Calculated from equation 8.
j. Calculated from equation 9.
k. Observed values from tables 1 and 2 of reference 8.
l. Calculated from equation 10.
m. Calculated from equation 11.
n. From reference 8.
o. Observed value from table 4 of reference 8.
p. Calculated from equation 12.
q. From table 3 of reference 8.
r. Calculated from equation 14.
s. Calculated from equation 15.
t. From reference 11.

RESULTS AND DISCUSSION

The equations (1–4) correlating the in vitro growth inhibition of mouse mammary adenocarcinoma cells (TA 3) by substituted 2,4-diaminopyrimidines from the work of Jonak et al, are given in table 1 where n is the number of compounds tested in each series, r is the correlation coefficient of the regression, and s is the standard deviation. Comparison of equations 1 and 2 indicates that Taft's steric constant (E_s) for the 5-position of pyrimidine gives a better correlation than the log P of the whole molecule (P being the octanol-water partition coefficient). When log P and E_s are used simultaneously, equation 3 is obtained, and the inclusion of the log P term in this equation is justifiable at the 90 percentile level as indicated by an F test ($F_{1,7} = 4.67$, $F_{1,7_{0.10}} = 3.57$). Equation 4 is a parabolic equation of log P in combination with the E_s term, and the $(\log P)^2$ term is also statistically significant at the 90 percentile level ($F_{1,5} = 4.17$, $F_{1,5_{0.10}} = 4.06$). The ideal lipophilic character ($\log P_o$) for maximum anticarcinoma activity can be calculated from equation 4 by taking the first derivative, $d \log(1/\mathrm{ID50})/d \log P$, setting this equal to zero, and solving for log P. For this set of data, log P_o is approximately 4.40. Because relatively few data points were included ($n = 9$), the 95% confidence interval for log P_o could not be defined. The negative coefficient associated with E_s in equation 4 suggests that a bulky substituent at the 5-position enhances the anticarcinoma activity of the diaminopyrimidines.

The quantitative results obtained for the 2, 4-diaminopyrimidines demonstrate that the anticancer activity is dependent on the steric nature of the substituent at the 5-position and the log P of the whole molecule. This agrees with the previous work of Baker whose results indicated that lipophilic substituents at position 5 of the diaminopyrimidines increase the binding of these compounds to pigeon liver dihydrofolate reductase because of hydrophobic interactions (15). Baker hypothesized that the hydrophobic region is adjacent to the active site but not part of it (15). Miller and Hansch (16) have shown the binding of a series of substituted pyrimidines and triazines to the same enzyme to be dependent on the electron-releasing and lipophilic character of the substituents. They suggested that large lipophilic substituents with negative σ^* values be incorporated in order to obtain more potent inhibitors.

The most interesting result of our analysis is the finding of negative dependence on E_s and parabolic dependence on log P (equation 4). Both of these parameters suggest the importance of hydrophobic interactions. The parabolic dependence on log P may be due to the bulk tolerance of the binding site (17). Of the 2, 4-diaminopyrimidines examined, the adamantyl derivative has the highest activity, although the log P value is about 0.7 units below log P_o.

Recently, Notari and his co-workers (18) did comparative studies on the kinetics of the hydrolytic deamination of cytosine nucleosides. They found that the 6-methyl derivative of cytosine arabinoside is chemically two to five times more stable to deamination than the parent compound cytosine arabinoside.[5] It has been shown that the 5′-adamantoate derivative of cytosine arabinoside is more active than cytosine arabinoside in L1210 leukemic mice where the duration of cytotoxic plasma levels of the parent compound is greatly increased by administration of the adamantoate derivative. It is possible that the 2,4-diaminopyrimidines may inhibit the enzymatic oxidative deamination of the cytosine nucleosides to their corresponding uracils.

Equations 5–7 are derived from the in vitro growth-inhibitory activity of N-acyltriamines related to solapalmitine in human nasopharynx carcinoma cell cultures reported by Kupchan and his co-workers (7). For the 28 homologs reported with definite ED50 values, the parabolic equation of π_{acyl} (equation 6) gives a slightly better correlation than the parabolic equation (equation 5) using log P for the whole molecule. However, the correlation coefficients ($r = 0.849$ versus $r = 0.784$) are lower than

[5] Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

the minimum acceptable value of 0.90.[6] When the methylene chain length (n) for the terminal tertiary amino groups is held constant at $n = 4$ and the acyl side chain length (m) is varied from 4 to 20, a parabolic equation of log P gives a good correlation (equation 7, $r = 0.943$) for 13 compounds. In this subgroup, the log P_o is approximately 8.56 for the undissociated form with a 95% confidence interval of 8.16–9.14. Since both of the tertiary amino groups will be protonated at physiologic pH, the real log P_o value will be approximately 6 log units[7] below the undissociated form. In other words, the log P_o value will be approximately 2.56 for the diprotonated species.

The results obtained for *N*-acyltriamines showed the importance of the acyl side chain in determining maximal cytotoxicity. Our results indicate that for the 28 compounds examined, the optimum π_o (acyl) value is 7.24 with a 95% confidence interval of 6.59–8.45. This corresponds to a 14–15-carbon acyl side chain. When the number of terminal tertiary amino side chains is held constant at 4, the optimum log P_o for the whole molecule is 8.56 which corresponds to a 16-carbon acyl side chain. This is in agreement with the original finding of Kupchan and co-workers that for maximum cytotoxicity an acyl residue at least 12 carbons in length is required (7).

For bis-(1-aziridinyl)-phosphinyl carbamates acting against Walker 256 carcinoma in rats, neither the summation of the π values of R and R', nor Taft's polar substituent constant σ^* for R' gives a reasonable correlation for the anticancer activity or toxicity (equations 8, 9, 11, and 12). However, σ^* for R alone gives good correlation for the anticancer activity (equation 10), but this parameter only slightly improves the correlation for the toxicity in rats (equation 13). In these two equations, the positive coefficient associated with $\sigma^*_{(R)}$ suggests that electron-withdrawing groups like F, Cl, $-CHF_2$, or $-CF_3$ may enhance the activity as well as the toxicity. Since the coefficient of equation 10 is one unit higher than that of equation 13, the activity should be increased to a greater extent than the toxicity. In other words, the therapeutic index may be increased by introducing electron-withdrawing groups on the aziridinyl ring.

For the aromatic nitrogen mustards against Walker 256 carcinoma in rats, equations 14–19 are obtained. The most striking feature of these equations is the negative dependence on the π value. These equations and equations 9 and 12 are in agreement with the finding of Hansch et al that the log P_o values for the antileukemic activity of nitrosoureas, another series of alkylating agents, are very low (-0.6). The negative dependence on σ (equations 14–16) suggests that electron-releasing groups will enhance the activity and toxicity. This may be attributed to the higher electron density which makes the nitrogen mustard a stronger base and forms an immonium ion more easily. Functional groups with negative π and σ values, such as 2,4–$(NH_2)_2$, 2,4–$(NCH_3)_2$, 2,4–$(OH)_2$, and 4–$NHNH_2$ may be useful in assessing the role of these parameters with a greater degree of confidence.

The relatively low value for π_o (0.34) of equation 18 suggests that a highly lipophilic group will decrease the activity as well as the toxicity (*see* fig 1). In figure 1 it can be seen that the best separation of the ED50 from the LD50 occurs at π values around 0.4 $\sim$ 0.5. In other words, the R'' of –OCONHPh(4–COOH) appears to be the best choice of the substituents studied.

Since many of the substituent constants are not available, values estimated from those of the homologs must be used. These may differ slightly from the real values. However, for our discussion this should not seriously affect the correlations obtained. Although some of the correlations are not quite as good as those reported for the much simpler antibacterial or

[6] The minimum acceptable value of 0.90 for the correlation coefficient (r) is a value fixed arbitrarily a priori for these types of quantitative structure-activity relationships. An "r value" equal to 0.90 statistically means that $r^2 = 0.81$ or 81% of the variance in the biologic activity (Y) is explained by the regression of Y on X.

[7] This is calculated as follows: $P_{\text{undissociated}} = \frac{P}{(1-\alpha)}$, so that $pK_a = 10.4$, pH = 7.4, and $\alpha = 0.999$, where α = the degree of dissociation, and, for the monoprotonated species, $P = 0.001 \times (P_{\text{undissociated}})$ or $\log P = \log P_{\text{undissociated}} - 3$. For the diprotonated species, $\log P = \log P_{\text{undissociated}} - 6$.

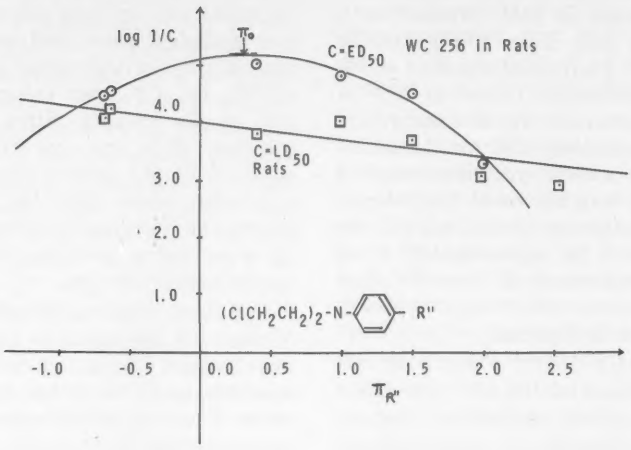


FIGURE 1.—Plot of log $1/C$ versus π_R'' for equations 18 and 19 indicating the relationship of ED50 and LD50 with respect to π_R''. WC 256 = Walker 256 carcinoma.

antifungal activities (19,20), it is gratifying to see that so many series of different compounds could be analyzed quantitatively in terms of substituent constants. Figure 2 depicts the plot of σ_R versus π_R for the functions included in equations 14 and 15. The scattering of the points indicates that the σ_R and π_R used are independent variables (21).

Drug design based upon multiple regression analysis and proper selection of substituents (22) could eliminate the needless synthesis and testing of many unpromising compounds and provide more rational guidelines for quantitative prediction of the activity of new cancer chemotherapeutic agents.

In order to test our working hypothesis, we will attempt to synthesize some of the compounds predicted to be more active than the existing ones. For example, bis-(1-aziridinyl)-phosphinyl carbamates with electron-withdrawing groups on the aziridinyl rings and aromatic nitrogen mustards bearing functional groups with negative π and σ values will be synthesized and tested for their anticancer activity.

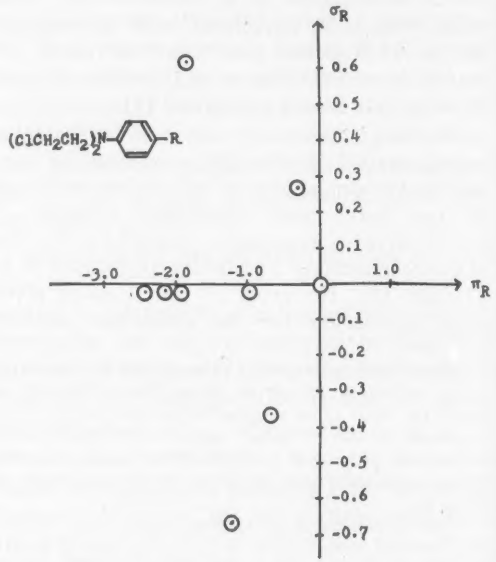


FIGURE 2.—Plot of σ_R versus π_R for equations 14 and 15 indicating the lack of interdependence between these two variables.

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Evaluation of 4-Carbethoxy-5-(3,3-dimethyl-1-triazeno)-2-phenylimidazole (NSC-166721) and 4-Carbethoxy-5-(3,3-dimethyl-1-triazeno)-2-methylimidazole (NSC-166722) as Potential Antitumor Agents [1,2]

James Heyes, Elaine J. Catherall, and Andrew Cockburn [3]

SUMMARY

The biologic evaluation of 4-carbethoxy-5-(3,3-dimethyl-1-triazeno)-2-phenylimidazole (BRL-51242) and 4-carbethoxy-5-(3,3-dimethyl-1-triazeno)-2-methylimidazole (BRL-51308) is reported. The antitumor activity of these compounds against a number of experimental rodent tumors is described and compared with that of the known triazenoimidazole 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388). The toxicity of BRL-51242 and BRL-51308 in several species is outlined and compared with that of NSC-45388.

[Cancer Chemother Rep 57:263–268, 1973]

Considerable interest has been shown in the antitumor activity associated with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) (1,2).[4] This compound has shown activity against a number of experimental neoplasms (2), and clinical trials carried out under the auspices of the National Cancer Institute have shown it to be a useful agent for the treatment of malignant melanoma in man (3–6). The dose-limiting toxicity is myelosuppression, although nausea and vomiting have also been troublesome.

Since the initial communication regarding the antitumor activity of NSC-45388 and some analogs in 1962 (1) it has been pointed out that these agents are analogs of 5-aminoimidazole-4-carboxamide,[5] which as a ribophosphate is an intermediate in the biosynthetic pathway to purine ribonucleotides. In attempts to improve the therapeutic potential of this material, modification of the imidazole ring has mainly centered around the 4- and 5-positions, ie, the ester and triazene groupings (7–10). Substitutions at the 2-position of the imidazole have been a notable exception to these modifications.

The antitumor and carcinogenic activities of a number of aryl-triazenes in rodents have been known for some years (11,12); recently, further interest has been shown in these materials (13,14).

In recent years we have investigated the biologic activity of a number of aminoimidazoles substituted at the 2-position, and we have now prepared the corresponding triazenoimidazoles. We report here the biologic evaluation of two of the more interesting compounds, 4-carbethoxy-5-(3,3-dimethyl-1-triazeno)-2-phenylimidazole (BRL-51242) and 4-carbethoxy-5-(3,3-dimethyl-1-triazeno)-2-methylimidazole (BRL-51308) (fig 1).

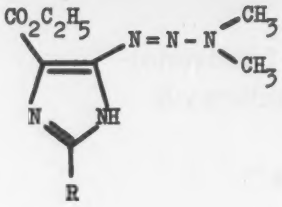
[1] Received Jan 9, 1973; revised May 17, 1973; accepted May 24, 1973.

[2] Agents used in this study were prepared by Mr. N. Ward, Beecham Research Laboratories.

[3] Beecham Research Laboratories, Brockham Park, Betchworth, Surrey, England.

[4] NSC-45388: CAS reg. No. 4342-03-4; DTIC.

[5] 5-Aminoimidazole-4-carboxamide, monohydrochloride: NSC-113496; CAS reg. No. 72-40-2; AIC.



BRL 51242 R = Phenyl

BRL 51308 R = Methyl

FIGURE 1.—Chemical structures of BRL-51242 and BRL-51308.

MATERIALS AND METHODS

Tumor Screens

Friend virus leukemia of mice.—This virus-induced murine leukemia causes progressive increase in spleen size, with death normally resulting from rupture of the spleen (15). The drug was given to groups of ten BALB/c mice by the subcutaneous (sc) route on Days −1, +1, +2, and +3 with respect to the intravenous (iv) administration of the virus inoculum on Day 0. The animals were killed on Day 14, and the spleens were weighed and compared with those of control animals.

R1 lymphoma.[6]—This mouse tumor normally responds to antimetabolites, but it rarely responds to alkylating agents. Routinely an inoculum of 10^6 ascitic cells was transplanted by sc injection into the groin of CBA mice; this resulted in the migration of the tumor cells to all parts of the body, followed by death about 10 days later. Drug treatment was carried out on Days 3–7 after transplantation in groups of ten mice, and the percentage increase in survival time of treated animals over that of control animals was measured.

[6] We are greatly indebted to Dr. T. A. Connors of the Chester Beatty Research Institute for his interest and help in setting up the R1 lymphoma and PC6 plasma cell experimental tumor systems at Beecham Research Laboratories.

ADJ/PC6A plasma cell tumor.[6]—This solid mouse tumor is sensitive to most alkylating agents. The tumor was transplanted sc into BALB/c mice and allowed to grow for about 21 days. Treatment was then carried out for 5 days in groups of ten mice. The animals were killed 5 days later, and the percentage inhibition of the tumor weight of treated animals compared with that of control animals was determined. The dose level which gave a 90% inhibition of the tumor weight (ID90) was then compared with the LD50 to give a therapeutic index.

Toxicity

An acute comparative study of oral and iv treatment with BRL-51242, BRL-51308, and NSC-45388 was carried out in mature male and female beagle dogs. One dog of each sex was used for each of the studies. The dose regimen was 25 mg/kg on Day 1, 45 mg/kg on Day 5, and a final dose of 90 mg/kg on Day 10. The hydrochloride salts of BRL-51242 and NSC-45388 were used for the iv doses since the bases were insoluble in water. BRL-51308 was very soluble in water and was used as the free base.

An acute oral study in the rat was carried out with BRL-51242 and NSC-45388 in groups of eight 8-week-old male Carworth CFY rats.

A 28-day repeat-dose study with BRL-51242 administered by the oral route was carried out in dogs and baboons. The drug was given to groups of four animals, two males and two females, at a dose of 10 mg/kg/day or 45 mg/kg/day.

Drugs

NSC-45388 was prepared according to the method of Shealy et al (9) from 5-aminoimidazole-4-carboxamide (16). BRL-51242 and BRL-51308 were prepared in a similar manner from the corresponding amines (17).

Melting points, solubility in water, and the results of elemental analysis of BRL-51242 and BRL-51308 are shown in table 1.

RESULTS

Antitumor Screens

The activity of BRL-51242 was first indicated by its action against Friend virus disease.

TABLE 1.—Results of elemental analysis, melting points, and solubility in water of BRL-51242 and BRL-51308

BRL-51242

Analysis: $C_{14}H_{17}N_5O_2$: calculated: C, 58.53; H, 5.92; N, 24.40
found: C, 58.26; H, 5.88; N, 24.45

Melting point: 171 C

Solubility in water at 20 C: < 0.1 mg/ml

BRL-51308

Analysis: $C_9H_{15}N_5O_2$: calculated: C, 48.00; H, 6.67; N, 31.10
found: C, 47.81; H, 6.81; N, 31.05

Melting point: 116 C

Solubility in water at 20 C: 700 mg/ml

At a dose of 250 mg/kg, BRL-51242 significantly reduced the spleen weight of mice infected with Friend virus ($P < 0.01$, fig 2). The spleens of infected mice had a mean weight of 650 mg, whereas the spleens of animals treated with BRL-51242 had a mean weight of 190 mg. The spleens of uninfected control mice weighed 180 mg. At lower doses, partial response was noted.

Determination of the range of activity of BRL-51242 and comparison with BRL-51308 and NSC-45388 was carried out with the R1 lymphoma and the ADJ/PC6A plasma cell tumor.

R1 lymphoma.—All of the drugs had good activity in this screen, as shown in figure 3. There was little difference between BRL-51242 and NSC-45388 in either the dose level at which maximum effect occurred (32 mg/kg) or the maximum extension of life (60%–75%). BRL-51308, however, gave significantly better maximum extension of life than did either of the other drugs, and it had good activity at lower dose levels. A positive control of methotrexate [7] gave a maximum increase in lifespan of 50% at an optimal dose level of 2 mg/kg.

The influence of the schedule of treatment on the activity of BRL-51242 in the R1 lymphoma was also investigated (table 2). It can be seen that daily dosing to death was more effective than treatment with a single large dose. Treatment with a number of doses spread

[7] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

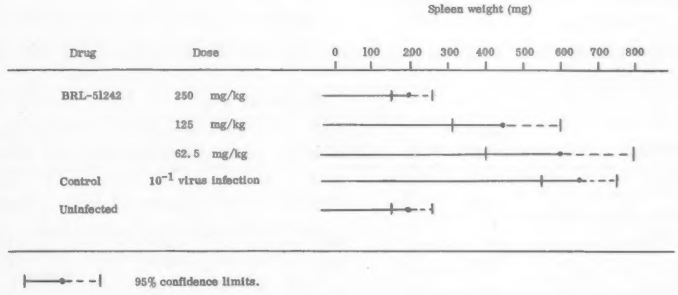


FIGURE 2.—Effect of BRL-51242 on the spleen weight of mice infected with Friend leukemia virus.

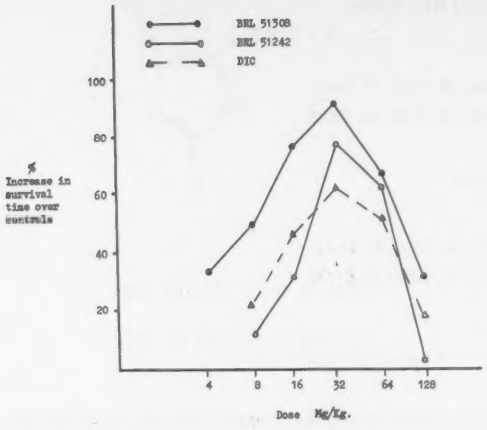


FIGURE 3.—Activity of BRL-51242, BRL-51308, and NSC-45388 (DIC) administered ip to mice with R1 lymphoma.

TABLE 2.—Influence of treatment schedule on the efficacy of ip administered BRL-51242 against the R1 lymphoma

Treatment schedule	Maximum increase in lifespan (%)	Optimal dose (mg/kg)
Daily × 5	80	32
Single dose	25	320
Five doses at 3-hr intervals	15	50
Daily doses to death	110	16
Every 4 days	45	128

over a single day or doses separated by 4 days also appeared to be less effective. A similar pattern of activity was found with BRL-51308.

PC6 plasma cell tumor.—All three drugs had good activity in this screen (table 3), whether administered intraperitoneally (ip) or orally. Again, BRL-51242 and NSC-45388 were similar in their activity. Although BRL-51308 had an LD50 similar to that of BRL-51242 and NSC-45388, the drug was active at lower levels.

Toxicity

Acute studies in the dog.—Oral or iv treatment with BRL-51242 at a dose of 25 mg/kg on Day 1, 45 mg/kg on Day 2, and 90 mg/kg on Day 10 caused no overt toxic effects, and the animals remained in good health for a prolonged period. Biochemical investigations showed increased serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) levels; no effects on the bone marrow were noted.

Iv treatment with BRL-51308 using the previously mentioned schedule caused vomiting and diarrhea in both dogs treated at the 90-mg/kg dose. The female dog subsequently remained in good condition for the duration of the study with no changes in hematologic values. However, a considerable reduction in platelet count and peripheral white blood cell count was noted with the male dog. No adverse clinical signs were noted after oral administration of BRL-51308. Tissue sections showed no microscopic evidence of any drug-induced change, and biochemical and hematologic values were normal.

TABLE 3.—Activity of BRL-51242, BRL-51308, and NSC-45388 against the PC6 tumor

Compound	Route of administration	Daily dose × 5 LD50 (mg/kg)	Daily dose × 5 ID90* (mg/kg)	Therapeutic index
BRL-51242	Ip	75	2.5	30
	Oral	150	10.0	15
NSC-45388	Ip	80	2.8	29
	Oral	110	6.5	17
BRL-51308	Ip	75	1.7	44
	Oral	110	2.4	46

*ID90 = dose at which tumor weight is inhibited by 90%.

Administration of NSC-45388 by either route using the previously mentioned schedule caused a massive decrease in the total number of white blood cells and platelets, and oral treatment of one dog had to be stopped after the second dose. Macroscopic examination of all dogs revealed enlarged hemorrhagic lymph nodes and considerable hyperemia of the duodenum, jejunum, and ileum.

Acute study in the rat.—A comparative acute study in the rat of oral treatment with BRL-51242 and NSC-45388 indicated that BRL-51242 (LD50, 2500 mg/kg) had an acute toxicity similar to that of NSC-45388 (LD50, 1750 mg/kg). Studies that included a detailed observation period showed that treatment with BRL-51242 gave rise to mild hepatotoxicity as indicated by a rise in SGOT and SGPT levels, whereas treatment with NSC-45388 caused a decrease in the number of circulating white blood cells.

Studies of repeated oral treatment with BRL-51242 in the dog and baboon (10- and 45-mg/kg/day doses) have indicated that the principal dose-limiting factor for this compound in these species is delayed hepatotoxicity. Biochemical analysis of routinely obtained blood samples showed high serum enzyme levels (SGPT, SGOT) at both dose levels. Sporadic vomiting was noted in the dog treated at 10 mg/kg/day 1–5 hours after administration of the dose. Regular vomiting occurred in both species after each dose at the higher level of 45 mg/kg.

DISCUSSION

The antitumor properties of these triazenoimidazoles are of particular interest since good activity against experimental tumors sensitive to alkylating agents as well as good activity against tumors sensitive to antimetabolites is not often seen in the same compound. The activities of BRL-51242 and BRL-51308 (in particular) compare very favorably with that of NSC-45388 in the tumor screens that we have investigated. The activity of orally administered BRL-51308 was encouraging and occurred at dose levels approaching that used for ip treatment, indicating that the drug was usefully absorbed.

The relative lack of bone marrow toxicity noted in the dog treated with our materials is also most encouraging, particularly since the main dose-limiting toxicity for NSC-45388 in both dogs and man is myelosuppression.

The difference in water solubility between BRL-51308, which is completely soluble, and BRL-51242 and NSC-45388, which are insoluble, would possibly indicate that their distribution in man could differ significantly. A different toxicity and anticancer spectrum might therefore be expected.

The antitumor and toxicologic properties of BRL-51242 and BRL-51308 are sufficiently encouraging to justify further investigation. BRL-51242 is now being evaluated against spontaneous tumors in dogs [8] and is undergoing an initial study in man.

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Cytotoxic, Antiviral, and Antitumor Activity of Some Derivatives of Daunomycin (NSC-82151) [1,2,3]

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L. Lenaz,[4,5] A. Necco,[4,5] and C. Soranzo [4,6]

SUMMARY

A number of daunomycin derivatives have been tested for cytotoxic, antiviral, and antitumor activity. The data in this paper support the hypothesis that modifications of the molecule of daunomycin can lead to substantial modifications in activity. Although cytotoxic and antiviral activity in vitro was partially retained with the derivatives, only 13-dihydro-daunomycin showed significant antitumor activity in vivo. A naturally occurring analog of daunomycin (daunosaminyl-daunomycin), with 2 moles of aminosugar per mole of aglycone, also displayed immunosuppressive activity which appears to be greater than that of the parent compound.

[Cancer Chemother Rep 57:269–274, 1973]

The antitumor antibiotic daunomycin is a clinically effective agent for remission induction in acute leukemia and for treatment of other kinds of malignancies (1,2). Its activity has been related to its ability to specifically interact with DNA (3). Adriamycin has been shown to be both as active as (4) and less active than (5) daunomycin in cell cultures in vitro, while its in vivo activity was shown to be higher than that of daunomycin in experimental tumors (6,7) and in clinical trials (8,9). The synthesis of new derivatives is of considerable interest for both increasing the therapeutic index and elucidating the structure-activity relationship. A number of daunomycin derivatives synthesized at the Farmitalia Research Institute, Milan, Italy (10–12), were tested for cytotoxic, antiviral, and antitumor activity in vitro and in vivo.

MATERIALS AND METHODS

In Vitro Studies

HeLa cells were maintained in Eagle medium supplemented with 10% calf serum. The mitotic activity was determined 8 hours after drug treatment. For determination of the effects on proliferative activity, the cells were exposed to the drugs for 8 hours, then the drugs were removed and normal medium was introduced. After 40 hours, cells were counted in an hemocytometer.

The antiviral activity was assayed according to the method of O'Connor (13) as previously described (14), with slight modifications. Secondary cultures of mouse embryo cells were plated on 35-mm Falcon plastic dishes, infected with the murine sarcoma virus (Mo-

[1] Received Feb 12, 1973; revised May 11, 1973; accepted May 24, 1973.

[2] Presented at the Symposium on Mechanism of Action of Cytostatic Agents, Budapest, Hungary, Oct 26–28, 1972.

[3] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

[4] Division of Experimental Oncology B, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy.

[5] On leave from Farmitalia Research Institute, Milan, Italy.

[6] The authors are indebted to Mrs. T. Comisso for her excellent technical assistance.

loney) (MSV-M), and treated for 3 days with the compounds under study. MSV-M was originally supplied by Dr. J. B. Moloney (National Cancer Institute, Bethesda, Md) and was then prepared from tumor tissue according to Moloney (15). The number of foci produced in the mouse embryo cultures was evaluated microscopically 5 days after the infection. Uninfected cultures were similarly treated and after trypsinization, cells were counted for determination of the effects of the compounds on cell proliferation.

In Vivo Studies

Ha/ICR mice of the CD_1 line (received from Charles River Breeding Laboratory, Paris, France, and then bred in our laboratory) were infected intramuscularly in the right hind leg with MSV-M and treated at different times after the infection. Tumor growth was graded on an arbitrary scale from +1 to +4 as previously described. Grade 5 was assigned to those animals that died with enlarged tumors. Animals that died because of toxicity were not considered (16).

Sarcoma 180 (S180) ascites cells were inoculated intraperitoneally (ip) into CD_1 mice (1×10^6 cells/mouse). Solid S180 was implanted subcutaneously by trocar in the flank of CD_1 mice. Gross leukemia originally induced by infection of newborn mice with the Gross leukemia virus was transplanted by intravenous (iv) inoculation of 2.5×10^6 cells from an homogenate of spleens and lymph nodes of leukemic mice in C3H/He Dp mice. The antitumor activity was established by the tumor weight index on the 11th day after implantation in the case of solid S180 and by the increase in average survival time for the other transplanted tumors. All compounds were dissolved in Ringer's solution and were administered ip or iv in a volume of 10 ml/kg of body weight.

Each experimental group consisted of at least ten animals. Each drug was tested over a wide range of doses. For the sake of brevity, only results obtained for the optimal dose will be included.

RESULTS

The chemical structures of daunomycin and its derivatives are shown in table 1. Some of the compounds being studied have changes in the acetyl side chain (R) in the 9-position of the saturated ring of daunomycinone: this group includes adriamycin, 13-dihydro-daunomycin, daunomycin-semicarbazone, daunomycin-thiosemicarbazone, and daunomycin-oxime. Other compounds show alterations in the aminosugar daunosamine; these derivatives are *N*-guanidine-acetamide-daunomycin, 2-amino-2-deoxy-glucosyl-daunomycinone, and an analog of daunomycin isolated in trace amounts from cultures of *Streptococcus peucetius*, containing 2 moles of daunosamine per 1 mole of daunomycinone, and tentatively identified as daunosaminyl-daunomycin.

In Vitro Studies

The 50% inhibiting doses (ID50) of these compounds on cell proliferation and on the foci formation induced by MSV-M are reported in table 2. The results confirm the high activity of daunomycin and adriamycin previously reported and show that the activity of daunomycin derivatives is lower in all systems than that of daunomycin; for all compounds, the effects on cell proliferation are higher in fibroblasts than in HeLa cells. All derivatives were more active in inhibiting MSV-M foci formation than cell proliferation, while daunomycin and adriamycin were almost equally active in the two processes. The ratio between ID50 on cell proliferation and on foci formation was therefore increased.

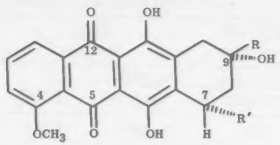
In Vivo Studies

All compounds were tested on a spectrum of experimental tumors (table 3). On MSV-M-induced rhabdomyosarcoma, daunomycin-oxime and daunomycin-semicarbazone, at the maximal tolerated dose, did not significantly affect tumor growth and regression; 13-dihydro-daunomycin, while not impairing tumor regression, was at least as active as daunomycin in inhibiting tumor growth.

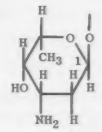
Daunosaminyl-daunomycin did not significantly affect the development of the tumor, but it prevented tumor regression in 80% of the treated mice; under the same experimental conditions, the regression of the tumor was

TABLE 1.—Chemical structures of daunomycin derivatives

General structure



Structure of daunosamine



Compound	Molecular weight	R	R'
Daunomycin •HCl	563.5	$-CO•CH_3$	Daunosamine
Adriamycin •HCl	580.0	$-CO•CH_2OH$	Daunosamine
Daunosaminyl-daunomycin •2 HCl	529.5	$-CO•CH_3$	Daunosaminyl-daunosamine
13-Dihydro-daunomycin •HCl	566.0	$-CHOH•CH_3$	Daunosamine
Daunomycin-semicarbazone	584.0	$-CH_3C=NNH•CONH_2$	Daunosamine
Daunomycin-thiosemicarbazone	600.0	$-CH_3C=NNHCSNH_2$	Daunosamine
Daunomycin-oxime •HCl	579.0	$-CH_3•C=NOH$	Daunosamine
N-Guanidine-acetamide-daunomycin •HCl	662.5	$-CO•CH_3$	*N*-Guanidine-acetamide-daunosamine
2-Amino-2-deoxy-glucosyl-daunomycinone •HCl	596.5	$-CO•CH_3$	2-Amino-2-α-D-deoxyglucose

TABLE 2.—Activity of daunomycin derivatives on cell proliferation and MSV-M foci formation in vitro

Compound	ID50 (μM)			
	Cell proliferation		MSV-M foci formation on MEF†(B)	A/B
	HeLa*	MEF† (A)		
Daunomycin •HCl	0.89	0.031	0.021	1.4
Adriamycin •HCl	1.9	0.034	0.020	1.7
Daunosaminyl-daunomycin •2 HCl	5.5	0.147	0.056	2.6
13-Dihydro-daunomycin •HCl	7.9	0.148	0.042	3.5
Daunomycin-semicarbazone	>8.5	0.171	0.042	4.0
Daunomycin-thiosemicarbazone	>8.3	0.216	0.070	3.0
Daunomycin-oxime •HCl	>8.6	0.690	0.093	7.4
N-Guanidine-acetamide-daunomycin •HCl	>7.6	0.150	0.113	1.3
2-Amino-2-deoxy-glucosyl-daunomycinone	>8.8	>0.167	>0.167	

*Treatment for 8 hours.
†MEF = mouse embryo fibroblasts; treatment for 72 hours.

TABLE 3.—Activity of daunomycin derivatives on a spectrum of experimental tumors

Compound	Activity at optimal dose					
	S180 solid	MSV-M sarcoma	S180 ascites		Gross leukemia	
	Tumor growth*	Tumor growth†	AST‡ (days)	LTS§	AST‡	LTS§
Daunomycin •HCl	50	63	222	6/11	176	0/10
Adriamycin •HCl	31	54	227	8/11	187	1/30
Daunosaminyl-daunomycin •2 HCl	53	88	195	4/10		
13-Dihydro-daunomycin •HCl	59	58	231	2/10	153	0/10
Daunomycin-semicarbazone	100	100				
Daunomycin-thiosemicarbazone			103	0/10		
Daunomycin-oxime •HCl		100	130	0/10		
N-Guanidine-acetamide-daunomycin •HCl			100	0/10		
2-Amino-2-deoxy-glucosyl-daunomycinone			107	0/10		

*%T/C tumor weight index at Day 11.
†%T/C ratio at maximal tumor volume (Day 10 after MSV-M inoculation).
‡AST = average survival time (control = 100).
§LTS = long-term survivors: No. surviving/No. treated.

abolished in only 30% of daunomycin-treated animals. In a series of experiments with control mice, more than 90% of the tumors undergo spontaneous regression (fig 1). Also, when administered below the infection, daunosaminyl-daunomycin prevented tumor regression in most animals; under these conditions the effects of this compound are similar to those of daunomycin (fig 2). When tested in solid and ascitic S180, the most active compounds were daunomycin, adriamycin, daunosaminyl-daunomycin, and 13-dihydro-daunomycin. The latter also retained significant activity in the transplanted Gross leukemia.

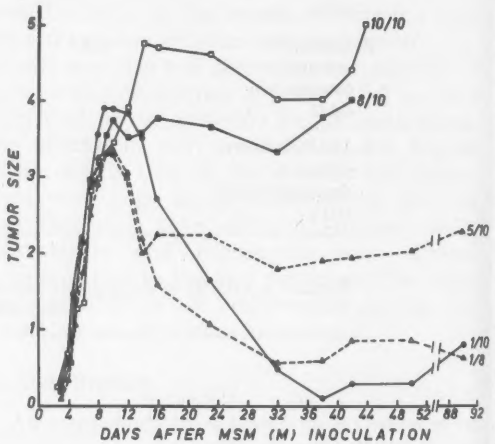


FIGURE 1.—Effect of daunomycin and daunosaminyl-daunomycin on MSV-M-induced tumors in mice. Treatment iv on Days 3–10 after the infection. On the abscissa: days after MSV-M infection. On the ordinate: tumor growth in arbitrary units (*see* Materials and Methods). Fractions on each curve: number of mice that died with enlarged tumor over total treated. ●———● = Controls; ▲———▲ = daunomycin (3.25 mg/kg/day); △———△ = daunomycin (2.6 mg/kg/day); ■———■ = daunosaminyl-daunomycin (4.2 mg/kg/day); □———□ = daunosaminyl-daunomycin (3.3 mg/kg/day).

DISCUSSION

The results obtained on HeLa cells in vitro correlate well with those previously reported on the binding ability of these compounds to DNA (3). In mouse embryo fibroblasts the effectiveness of all compounds was higher than that observed in HeLa cells, probably because of the longer period of exposure to the drugs. This prolonged period of treatment might also explain the relatively increased activity of adriamycin with reference to daunomycin, owing to the reported slower uptake of adriamycin by in vitro cultured cells (5).

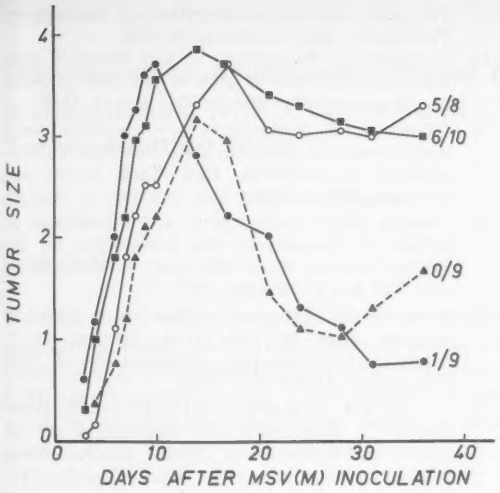


FIGURE 2.—Effect of daunomycin and daunosaminyl-daunomycin on MSV-M-induced tumors in mice. Treatment on Days 3–1 before the infection by the sc route. Fractions and coordinates as in figure 1. ●——● = Controls; ○——○ = daunomycin (7.5 mg/kg/day); ▲——▲ = adriamycin (5.2 mg/kg/day); ■——■ = daunosaminyl-daunomycin (7.5 mg/kg/day).

In general, the activity of the derivatives, although low on cell proliferation, was relatively higher on foci formation. It has been described (17) that some of these compounds are able to inhibit the reverse transcriptase activity of oncogenic RNA viruses, probably by binding to the template. The higher activity on MSV-M foci formation than on cell proliferation could suggest a higher specificity for the MSV-M template. When tested in vivo in the MSV-M-induced rhabdomyosarcoma, however, daunomycin-oxime and daunomycin-semicarbazone did not show any significant activity; this lack of effect, confirmed by the results obtained in other experimental tumors, could probably be accounted for by limited uptake by the cells and/or by rapid excretion. In all systems tested, 13-dihydro-daunomycin retained significant activity which, however, was generally inferior to that of the parent compound. This result is of particular interest since 13-dihydro-daunomycin, or daunorubicinol, was described as the major metabolite of daunomycin in mammals, and was supposed to have a higher activity than daunomycin (18). It must be mentioned, however, that the compound we tested was chemically prepared, and therefore it might have a different stereochemical configuration than daunomycin metabolites. Although it was rather active in solid and ascites S180, daunosaminyl-daunomycin displayed a peculiar effect on MSV-M-induced tumors. The MSV-M-induced tumor represents an interesting model for studying the immunodepressive activity of antitumor agents, because after an initial rapid growth it undergoes a spontaneous regression which has been shown to be due to immunologic mechanisms (16,19). The activity of daunosaminyl-daunomycin in preventing tumor regression is higher than that of daunomycin when it is administered after the infection; when administered before the infection, tumor regression was abolished in most of the mice, as in the case of daunomycin. Whether the strong immunodepressive activity of daunosaminyl-daunomycin is due to the inhibition of the immunologic mechanisms operating in the spontaneous tumor regression, such as immune lymphoid cells or cytotoxic or antiviral antibodies as observed for cyclophosphamide (20) and daunomycin (16), or to an increase in the synthesis and activity of blocking antibodies (21), remains to be elucidated.

Although none of the derivatives studied so far appear to be superior to the parent compound, the retained cytotoxic and antitumor activity of some of these compounds and the increased ratio between antiviral and cytotoxic activity versus daunomycin in vitro are of particular interest. Additional modifications are being undertaken with the goal of separating toxic and therapeutic properties within this important group of antitumor antibiotics.

LIST OF COMPOUNDS

Adriamycin: NSC-123127

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Daunomycin: NSC-82151; CAS reg. No. 23541-50-6; daunorubicin (NSC-83142)

Daunomycin-oxime: NSC-143491; daunomycin, 3-oxime, monohydrochloride, sesquihydrate

Daunomycin-semicarbazone: NSC-143114; daunomycin, 3-semicarbazone, monohydrochloride, dihydrate

Daunosaminyl-daunomycin: NSC-140781; daunomycin, daunosaminyl-, dihydrochloride

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Disposition of Arabinosylcytosine (NSC-63878) and 6-Thioguanine (NSC-752) in Solid L1210 Leukemia Tumor-Bearing Mice [1,2,3]

Robert F. Pittillo and Carolyn Woolley [4,5]

SUMMARY

Differential microbiological assay procedures were used for measuring tissue and tumor concentrations of arabinosylcytosine (ara-C) and 6-thioguanine given individually and in combination to BDF_1 mice bearing solid L1210 tumors. When the drugs are given in combination, detectable levels of ara-C in blood and tumor persist more than 1 hour longer than in mice receiving ara-C alone. 6-Thioguanine concentrations, when the drug is given in combination with ara-C, are decreased in the lung, brain, spleen, and urine, and are slightly increased in the liver. These findings may offer some possible explanation for the synergistic chemotherapeutic activity of these two agents in combination without enhanced toxicity.

[Cancer Chemother Rep 57:275–289, 1973]

Considerable interest has developed in the simultaneous use of arabinosylcytosine (ara-C) and 6-thioguanine. As a consequence of the report by Burchenal and Dollinger that an additive therapeutic effect of ara-C and 6-mercaptopurine[6] could be achieved without additive toxicity in mice with L1210 leukemia (1), Gee et al (2) evaluated the combination of ara-C and 6-thioguanine in the treatment of adult acute leukemia. They reported the combination to be an effective one for the induction of remission in 21 of 38 patients. Schmidt et al (3) studied the combination of ara-C and 6-thioguanine in normal BDF_1 mice and in mice injected intravenously (iv) with L1210 leukemia. High proportions of cures were obtained in mice challenged with 10^6 L1210 cells implanted iv when they were treated with ara-C plus 6-thioguanine every 3 hours on Days 1, 5, 9, and 13. They concluded that the benefits of the combination appeared to reside in the reduction of toxicity of 6-thioguanine when it was given simultaneously with ara-C.

Using microbiological assays, we have studied the physiologic disposition of ara-C and 6-thioguanine given alone or in combination to BDF_1 mice bearing solid L1210 tumors.

[1] Received Feb 12, 1973; revised June 14, 1973; accepted June 21, 1973.

[2] Supported by contract PH43-65-654 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Cytosine arabinoside: CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C.

6-Thioguanine: CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-.

Drugs used in this study were supplied by Drug Research and Development, DCT, NCI.

[4] Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Ala.

[5] The advice and support of Drs. J.A.R, Mead and L.B. Mellett are gratefully acknowledged. The technical assistance of Miss Sherry Burris, Mrs. Diane B. Graveman, and Mr. Stephen Chappell are appreciatively noted.

[6] 6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate.

MATERIALS AND METHODS

Microbiological assays of ara-C have been described (4,5). However, the sensitivity of the assay has been increased by the following means. *Streptococcus faecalis* ATCC 8043 resistant to 1 mg of actinobolin/ml and designated *S. faecalis*/ACB is uniquely sensitive to inhibition by ara-C. It is maintained on agar stabs of folic acid assay medium (Difco) supplemented with 1 mg of actinobolin/ml. This medium was used exclusively for this assay. For inocula, broth cultures of *S. faecalis*/ACB were grown for 18 hours at 37 C in the medium previously described, supplemented with 10 μg of actinobolin/ml. Cells from these cultures were collected and washed twice by centrifugation in saline (0.85% NaCl), resuspended in saline, and adjusted to 20% light transmittance (660 mμ) in a Spectronic-20 colorimeter (Bausch and Lomb, Inc., Rochester, NY). For the preparation of the assay medium, 37.5 g of folic acid assay medium was dissolved in 1 liter of distilled water and the pH adjusted to 6.0. After the addition of 1.5% agar, the medium was sterilized for 10 minutes at 15 pounds pressure. The medium was cooled to 50 C and supplemented with 0.5 μg of folic acid/ml and 10 μg of actinobolin/ml. One ml of the previously described cell suspension was added to 1 liter of agar; 6 ml was dispensed into the Petri plates (100 × 15 mm; Lab-Tex Products, Division of Miles Laboratories, Inc., Westmont, Ill).

A strain of *Escherichia coli* derived from *E. coli* ATCC 9637 as resistant to 1 mg of *dl-p*-fluorophenylalanine/ml (designated *E. coli*/FPA) was selected as the assay organism for 6-thioguanine because of its unusual sensitivity to inhibition by 6-thioguanine. The culture is maintained on agar slants of a glucose-salts medium (6) supplemented with 1 mg of *dl-p*-fluorophenylalanine/ml. For the preparation of seeded agar plates, stationary cultures were grown for 16–18 hours at 37 C in the glucose-salts medium. Cells from these cultures were collected and washed by centrifugation in saline and adjusted to 20% light transmittance as described for *S. faecalis*/ACB. The suspension was diluted 1:200 in saline, and 10 ml was added to 1 liter of cooled (50–55 C) glucose-salts medium; 6 ml of the inoculated agar was added to the previously described plates. All disc assay plates were held at 4 C for 24 hours prior to incubation at 30 C for 16–18 hours. This was essential to the increased sensitivity of the assays.

BDF_1 mice were inoculated subcutaneously with a spleen brei containing 10^6 cells/ml of L1210 leukemia solid tumor. The test mice were held for 10 days prior to single-dose intraperitoneal (ip) administration of 100 mg/kg of ara-C and 60 mg/kg of 6-thioguanine simultaneously. Each drug was also administered independently to other groups of mice. Immediately and at 15, 30, 60, and 120 minutes after injection, the tumors and tissues were removed, weighed, and prepared for assay. Each organ was homogenized with 1 ml of saline in a Sorvall Omni-Mixer Homogenizer Chamber (Ivan Sorvall, Inc., Norwalk, Conn). Blood samples were obtained by cardiac puncture immediately after sacrifice.

RESULTS AND DISCUSSION

Microbiological standard inhibition assay curves obtained for ara-C dissolved in saline (0.85% NaCl) and in freshly collected heparinized mouse blood and diluted with saline are presented in figure 1. Similar curves for 6-thioguanine are presented in figure 2. Standard curves were also prepared separately for ara-C and 6-thioguanine added to homogenates of liver, lung, spleen, brain, and urine. These standard curves are identical to those obtained when the drugs are dissolved in whole blood. Representative examples are given in figures 3 and 4. It should be noted that *S. faecalis*/ACB is resistant to 6-thioguanine at concentrations of 1250 μg/ml, and *E. coli*/FPA is resistant to ara-C at concentrations of 1250 μg/ml, which provide us with essential tools for differential measurement of each of these drugs when administered in combination. However, as a control test, all unknown samples obtained from individual and combination drug therapy in the mice were assayed against both organisms. No zones of inhibition were obtained from the ara-C standard solutions or samples from mice treated with ara-C when assayed against the 6-thioguanine assay organism and vice versa.

Graphically, figures 5, 6, and 7 illustrate the average levels and ranges of drug detected in blood, tumors, and tissues of L1210-bearing mice given a single ip dose of 100 mg/kg of ara-C (<0.04 LD10). Highest drug concentrations in the tumors and tissues were found approximately 60 minutes after drug administration (the exception being the urine concentrations which peaked at 30 minutes). Measurable quantities of ara-C were present for 2 hours in the lung and urine after drug administration.

It may be noted that the concentrations of ara-C detected in whole blood are lower than those found in serum by Mellett (7). The reason for this difference is not clear, but we have confirmed this observation in a series of separate experiments in which ara-C was dissolved in whole blood and in serum, and was then bioassayed. Approximately ten times more ara-C can be recovered from serum than from whole blood. It is possible that ara-C, like 6-thioguanine, is rapidly absorbed into the blood cells. Studies relating to this approach are in progress.

Ara-C concentrations detected in blood, tumors, and tissues of L1210-bearing mice given single ip doses of 100 mg/kg of ara-C and 60 mg/kg of 6-thioguanine simultaneously are presented in figures 8, 9, and 10. Blood and tumor levels of ara-C last more than 60 minutes longer in mice receiving ara-C and 6-thioguan-

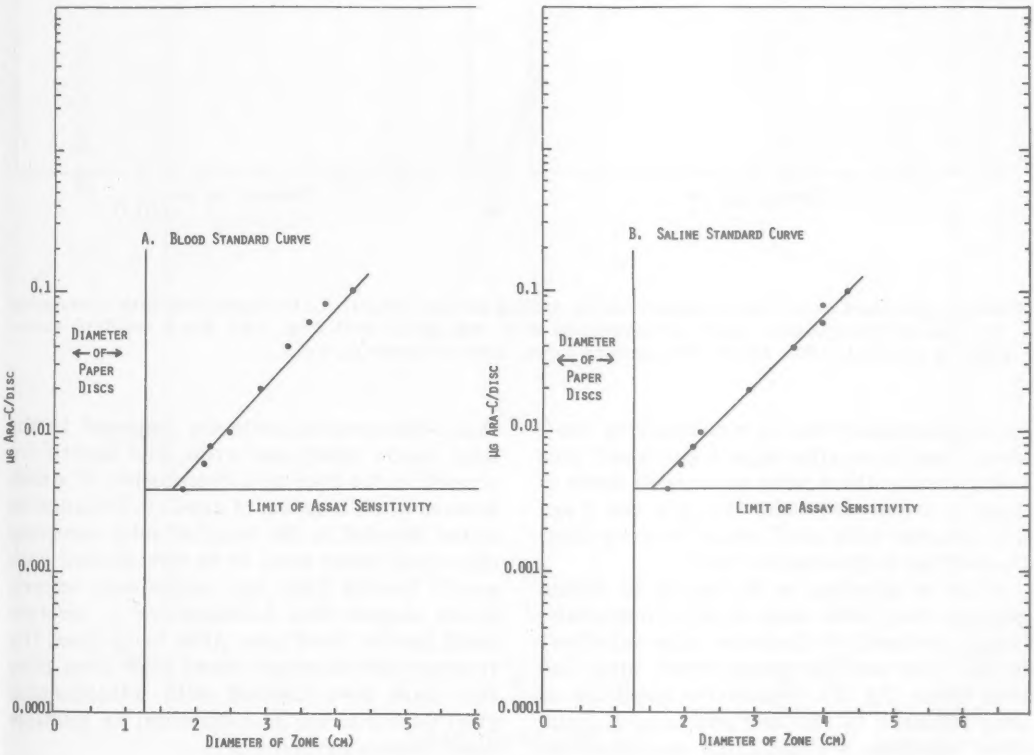

FIGURE 1.—Standard assay curves (constructed by method of least squares) for the logarithmic-ratio microbiological assay of ara-C. Assay microorganism is *S. faecalis* ATCC 8043/actinobolin. (*A*) Blood standard curve; index of precision 0.029. (*B*) Saline standard curve; index of precision, 0.029.

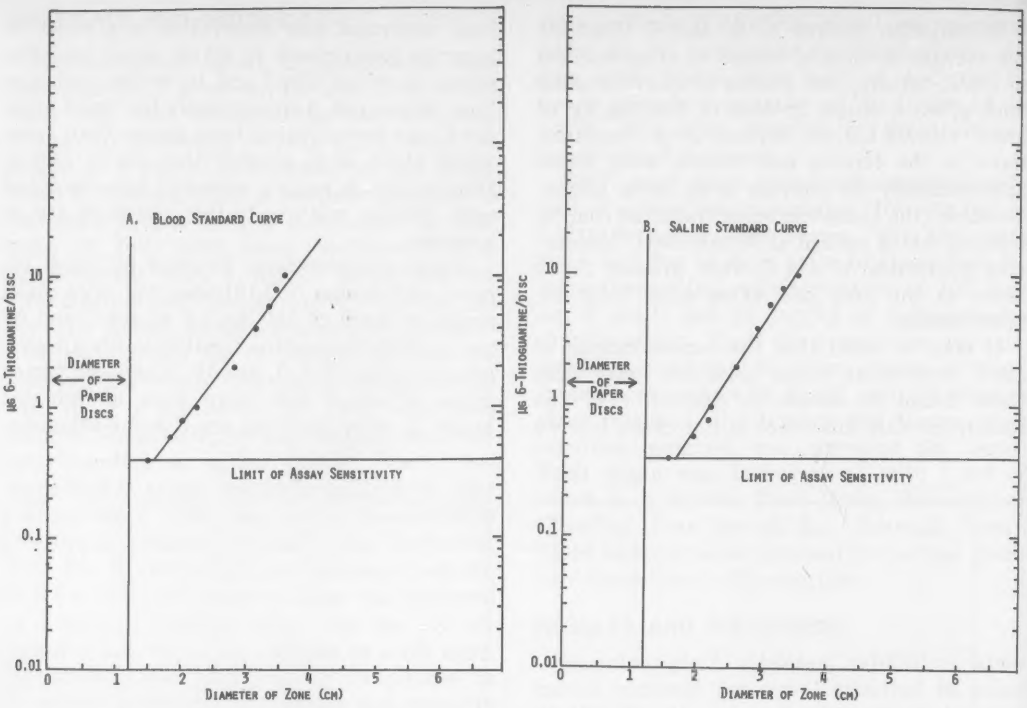

FIGURE 2.—Standard assay curves (constructed by method of least squares) for the logarithmic-ratio microbiological assay of 6-thioguanine. Assay microorganisms is *E. coli* ATCC 9637/FPA. (*A*) Blood standard curve; index of precision, 0.024. (*B*) Saline standard curve; index of precision, 0.015.

ine simultaneously than in mice receiving ara-C alone. Two hours after injection of ara-C plus 6-thioguanine, there were measurable levels of drugs in tumor and blood (30 μg/g and 1 μg/ml), whereas with ara-C alone the drug (ara-C) could not be detected at 2 hours.

After ip injection of 60 mg/kg of 6-thioguanine, drug levels were obtained immediately (approximately 1–2 minutes after injection) in the urine and the spleen, brain, lung, and liver tissue (fig 11). Measurable quantities of drug remained in the lung and urine 2 hours after injection. 6-Thioguanine concentrations in L1210-bearing mice given single ip doses of 60 mg/kg of 6-thioguanine and 100 mg/kg of ara-C simultaneously are presented in figure 12. It may be noted in these same mice (fig 10) that 6-thioguanine levels are decreased in the lung, brain, spleen, and urine, and slightly increased in the liver as a consequence of simultaneous administration of ara-C. 6-Thioguanine is not detected in the blood of mice receiving this agent either alone or in combination with ara-C. Results from our preliminary experiments suggest that 6-thioguanine is incorporated into the blood cells. After being lysed (by freezing and thawing), blood cells from mice that have been injected with 6-thioguanine yield 6-thioguanine as determined by multiple paper chromatography.

We can offer no biochemical or physiologic explanation for the results at this time. However, the marked persistence of ara-C in the tumors when given in combination with 6-

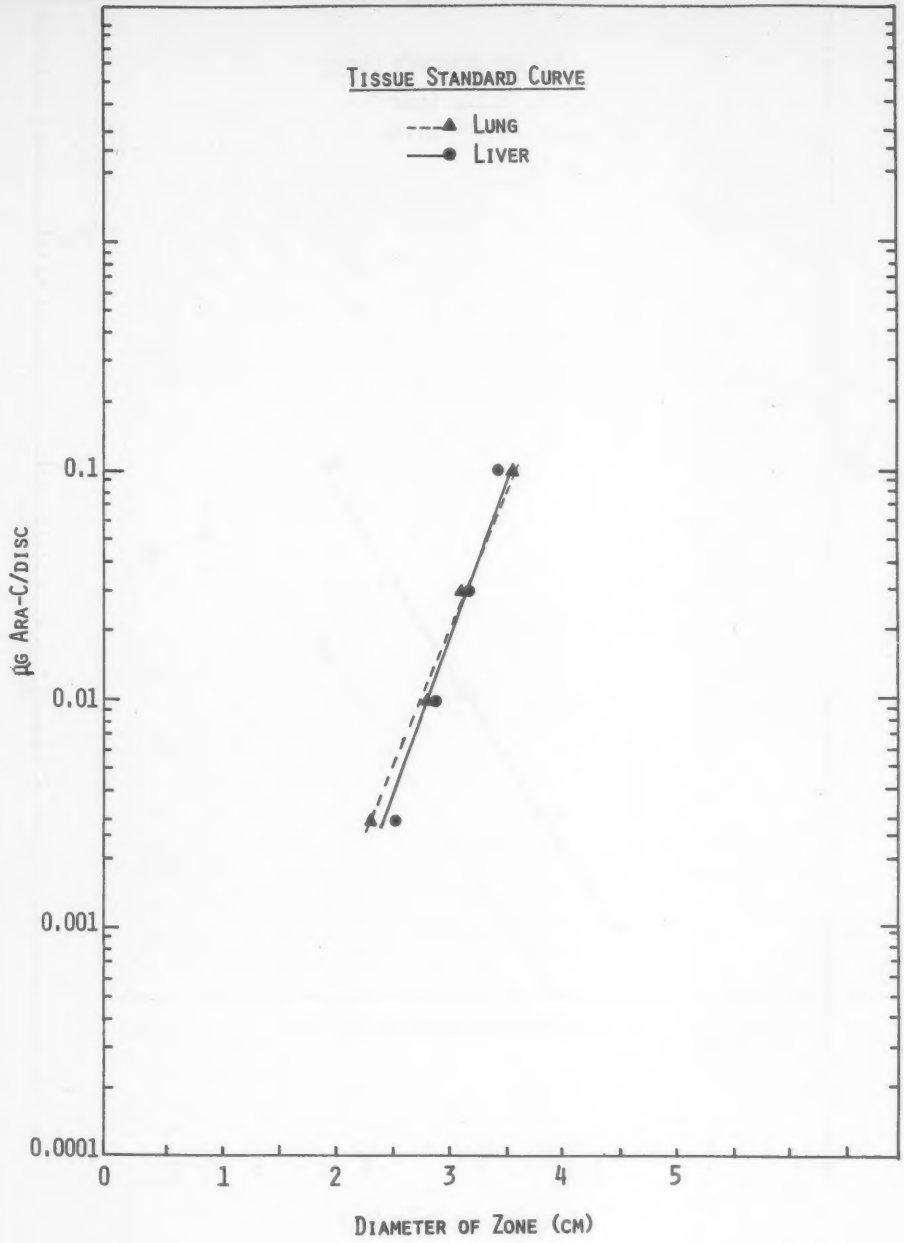

FIGURE 3.—Tissue assay curves of ara-C dissolved in lung and/or liver homogenates (constructed by method of least squares) for the logarithmic-ratio microbiological assay of ara-C.

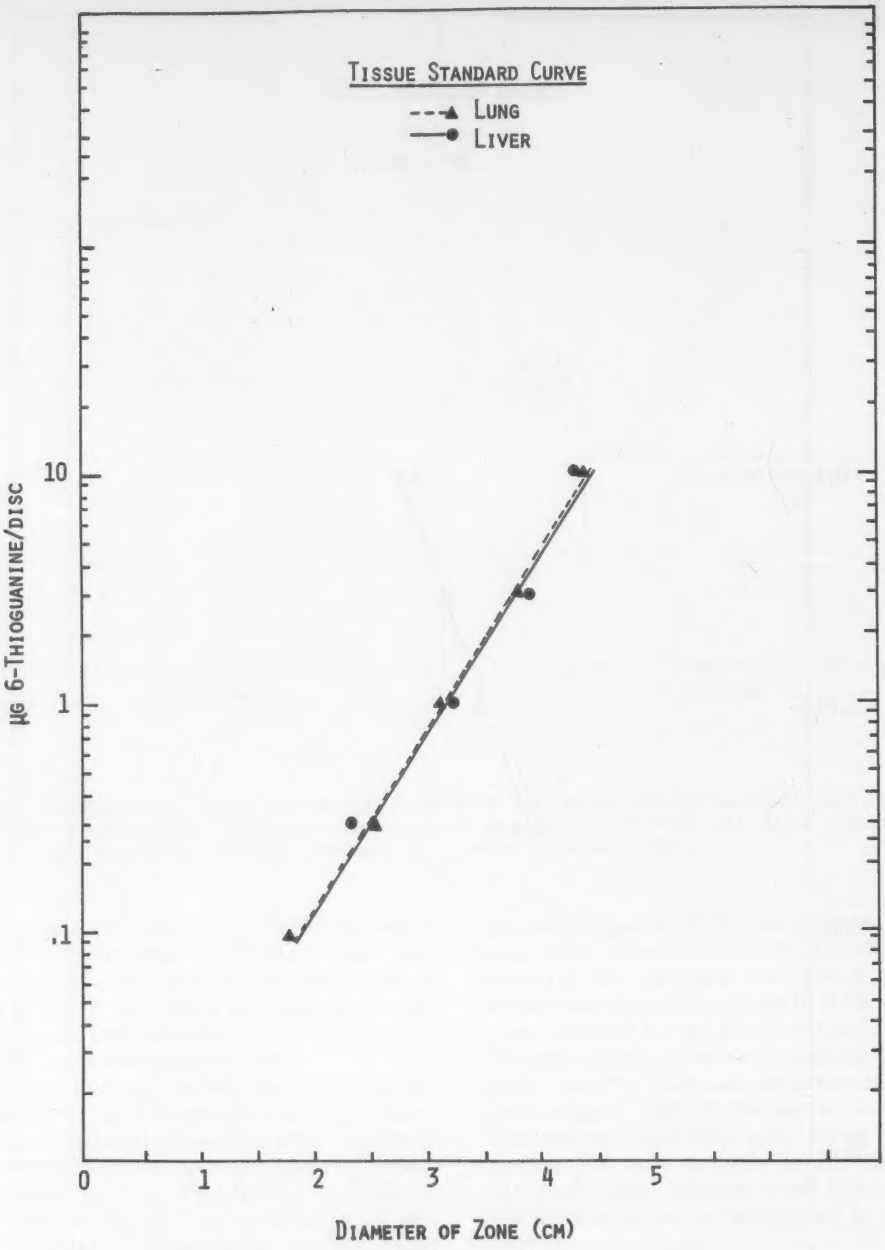

FIGURE 4.—Tissue assay curves of 6-thioguanine dissolved in lung and/or liver homogenates (constructed by method of least squares) for the logarithmic-ratio microbiological assay of 6-thioguanine.

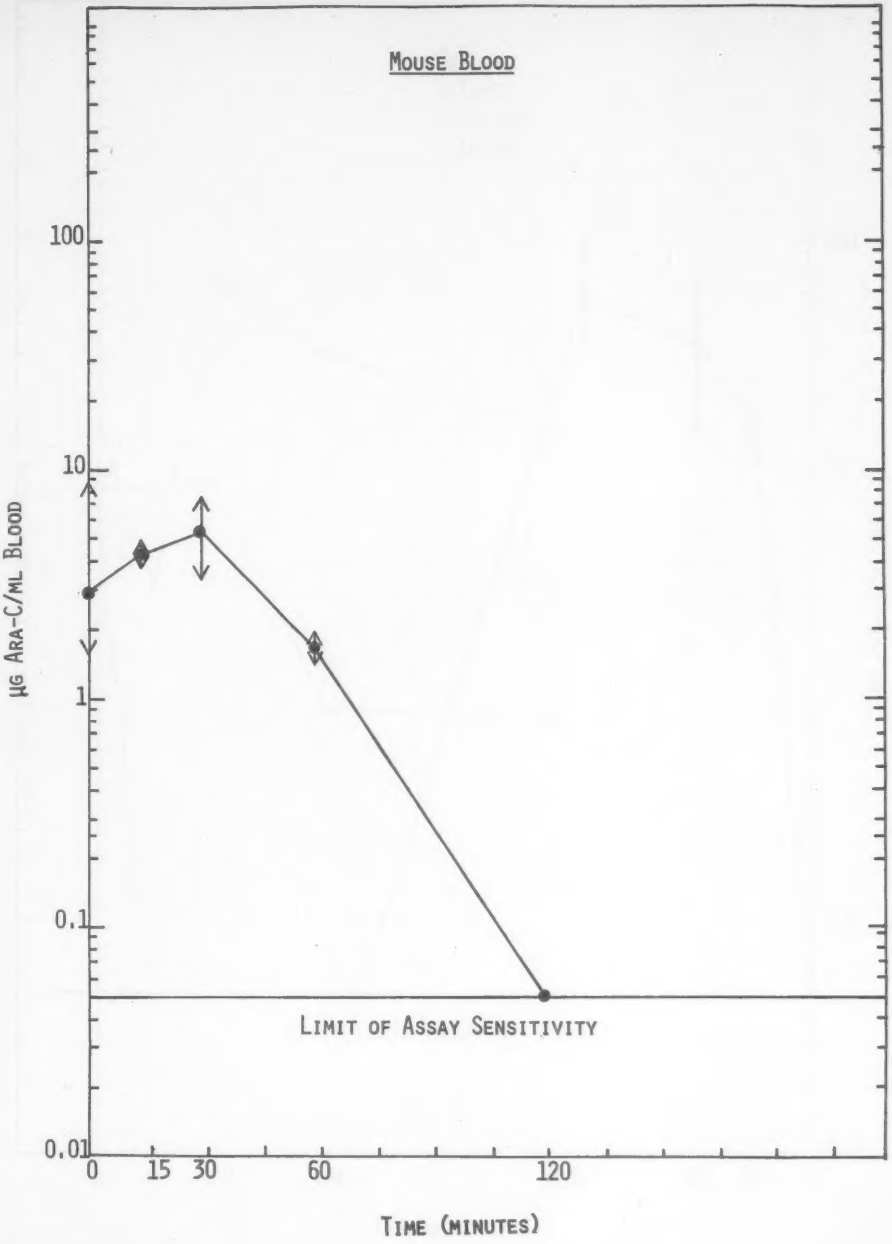

FIGURE 5.—Concentrations of ara-C (or cytotoxic equivalents) detected in blood of L1210-bearing mice by microbiological assay. Each point represents the average drug level of 5 mice and arrows refer to the individual range. The assay microorganism was *S. faecalis* ATCC 8043/actinobolin. Mice were injected with a single ip dose (100 mg/kg) of drug.

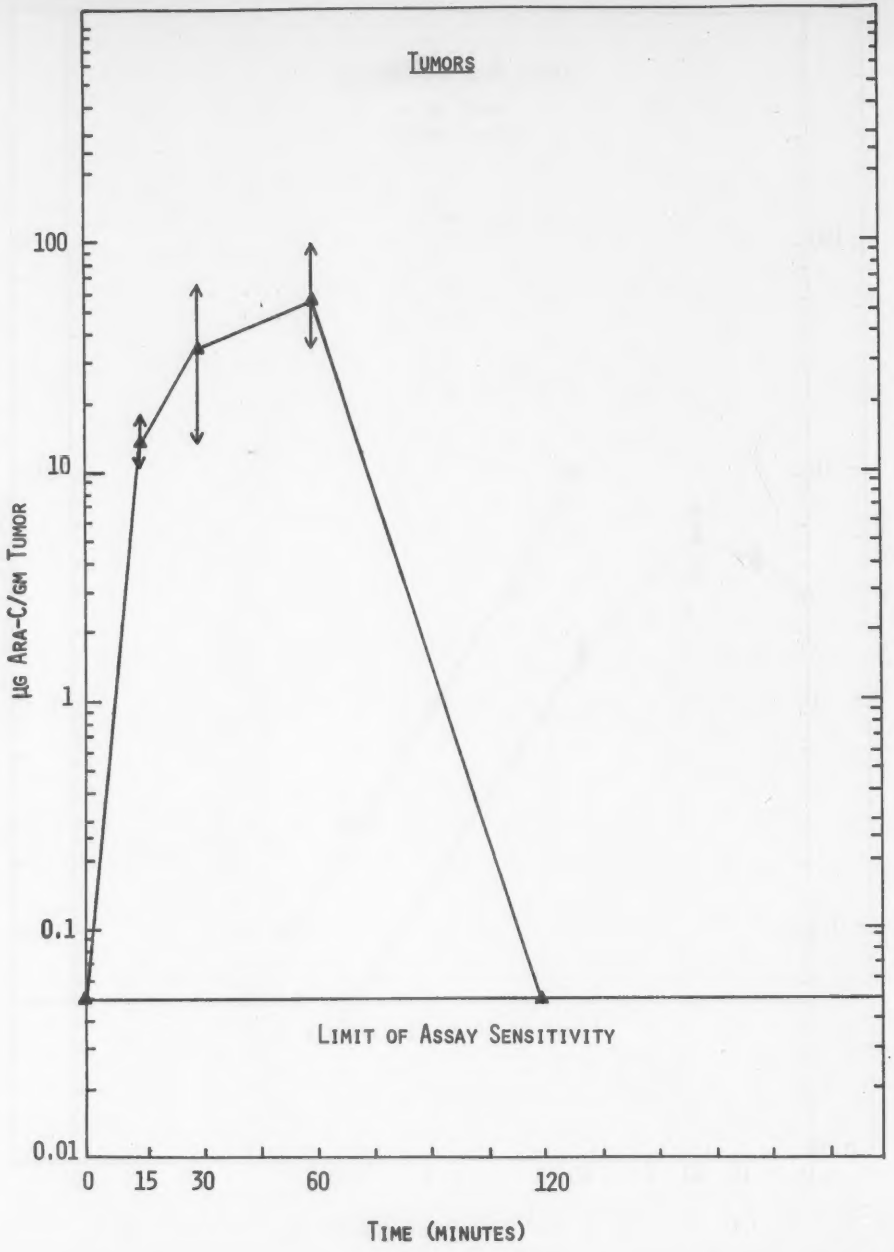

FIGURE 6.—Concentrations of ara-C (or cytotoxic equivalents) detected in tumors of L1210-bearing mice by microbiological assay. Each point represents the average drug level of 5 mice and arrows refer to the range of individual mice. The assay microorganism was *S. faecalis* ATCC 8043/actinobolin. Mice were injected with a single ip dose (100 mg/kg) of drug.

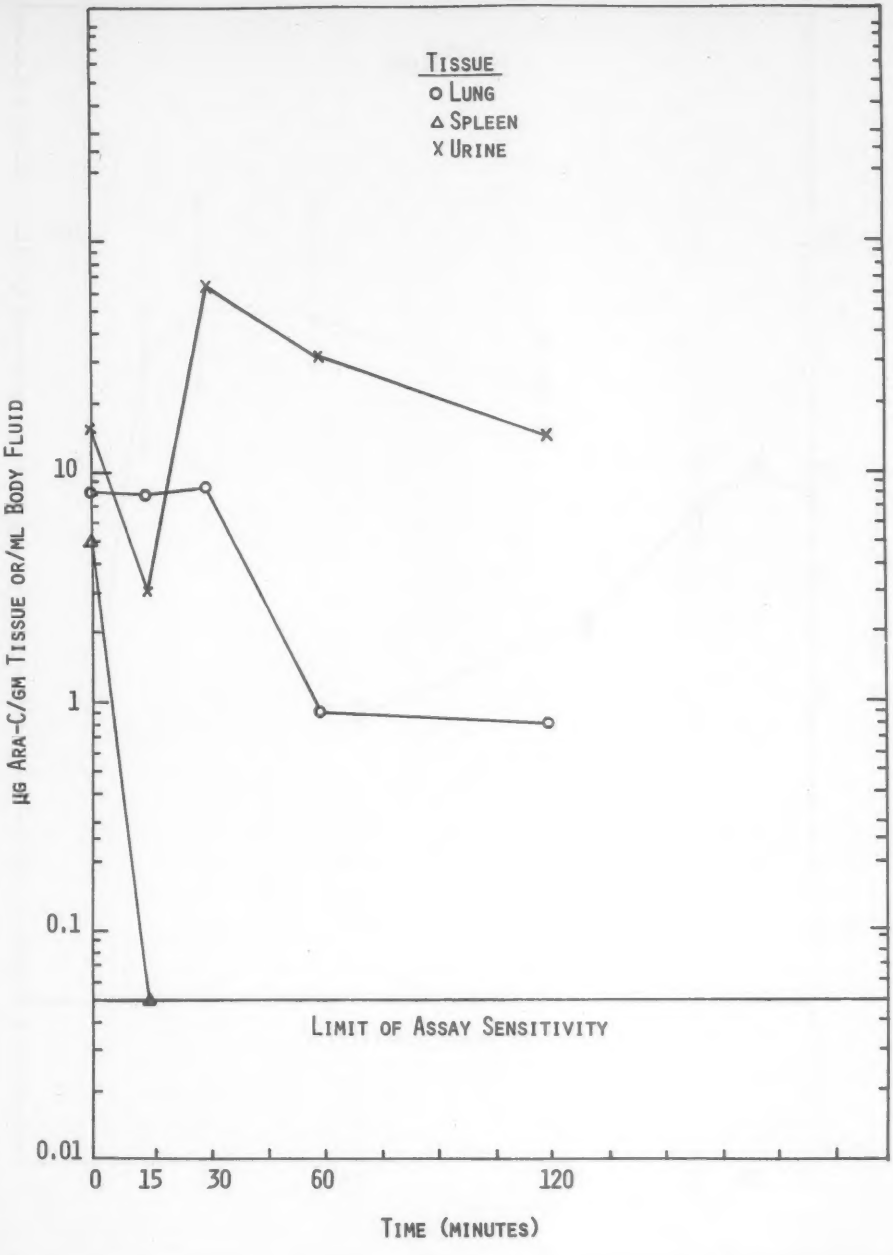

FIGURE 7.—Concentrations of ara-C (or cytotoxic equivalents) detected in tissues of L1210-bearing mice by microbiological assay. Each point represents the average drug level of 5 mice. The assay microorganism was *S. faecalis* ATCC 8043/actinobolin. Mice were injected with a single ip dose (100 mg/kg) of drug.

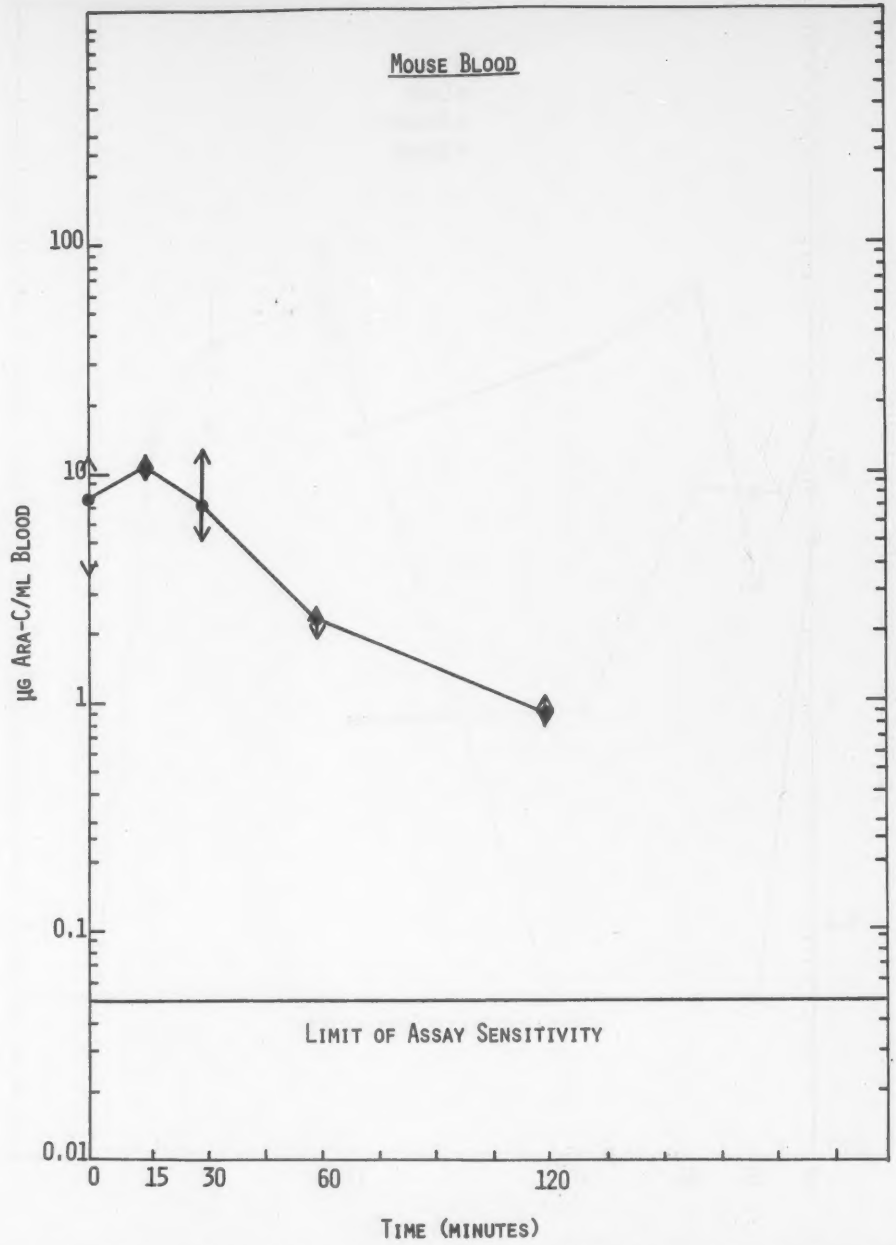

FIGURE 8.—Concentrations of ara-C (or cytotoxic equivalents) in combination with 6-thioguanine detected in blood of L1210-bearing mice by microbiological assay. Each point represents the average drug level of 5 mice and arrows refer to the range of individual mice. The assay microorganism was *S. faecalis* ATCC 8043/actinobolin. Mice were injected with a single ip dose of 100 mg/kg of ara-C and 60 mg/kg of 6-thioguanine simultaneously.

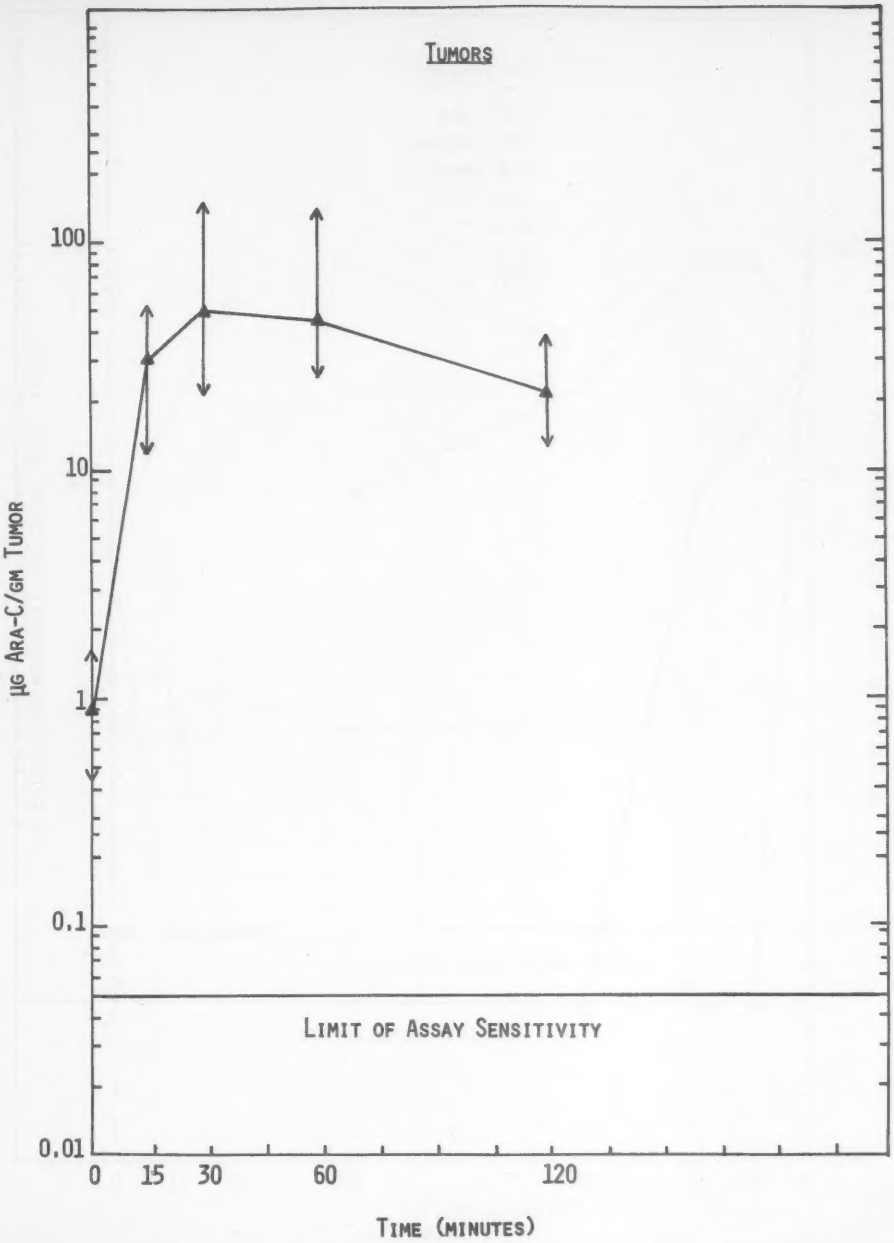

FIGURE 9.—Concentrations of ara-C (or cytotoxic equivalents) in combination with 6-thioguanine detected in tumors of L1210-bearing mice by microbiological assay. Each point represents the average drug level of 5 mice and arrows refer to the range of individual mice. The assay organism was *S. faecalis* ATCC 8043/actinobolin. Mice were injected with a single ip dose of 100 mg/kg of ara-C and 60 mg/kg of 6-thioguanine simultaneously.

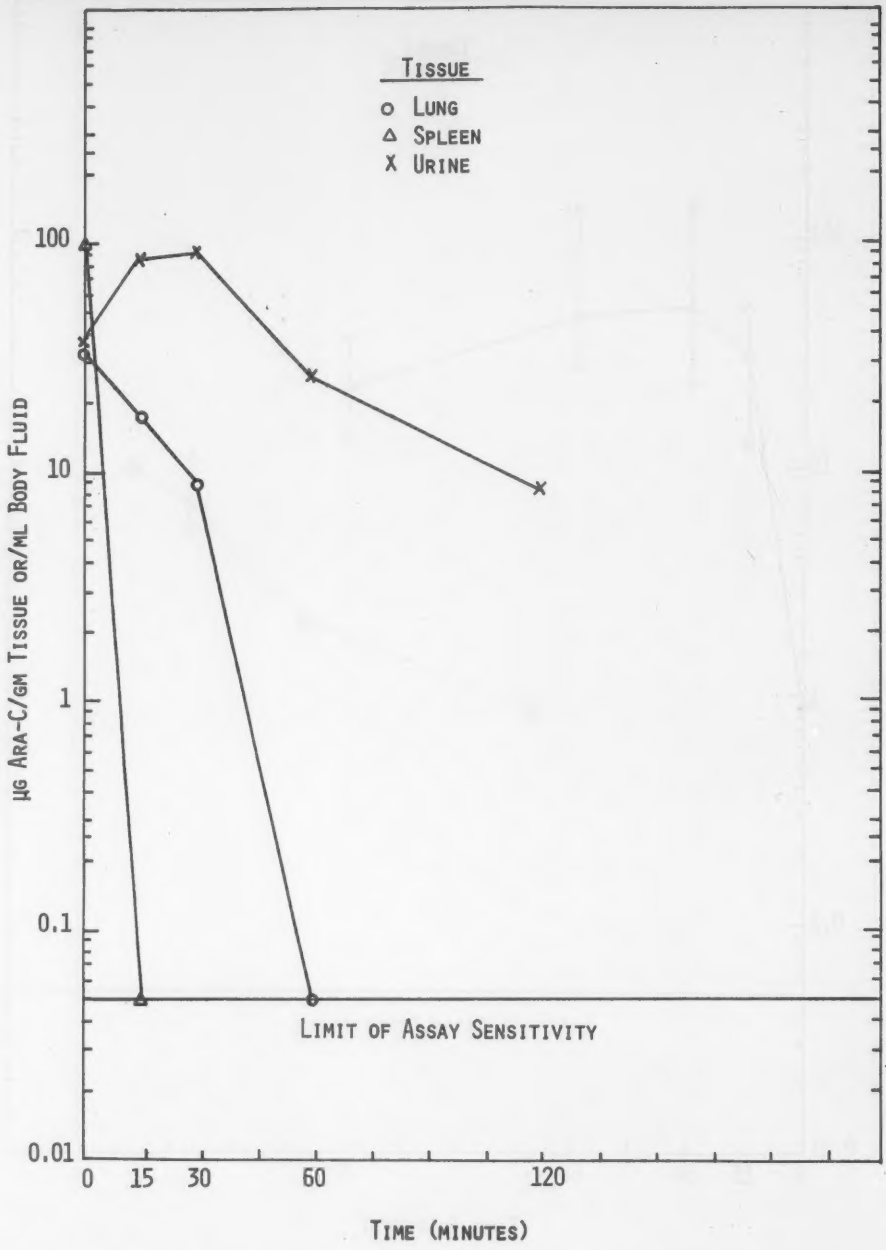

FIGURE 10.—Concentrations of ara-C (or cytotoxic equivalents) in combination with 6-thioguanine detected in tissues of L1210-bearing mice by microbiological assay. Each point represents the average drug level of 5 mice. The assay microorganism was *S. faecalis* ATCC 8043/actinobolin. Mice were injected with a single ip dose of 100 mg/kg of ara-C and 60 mg/kg of 6-thioguanine simultaneously.

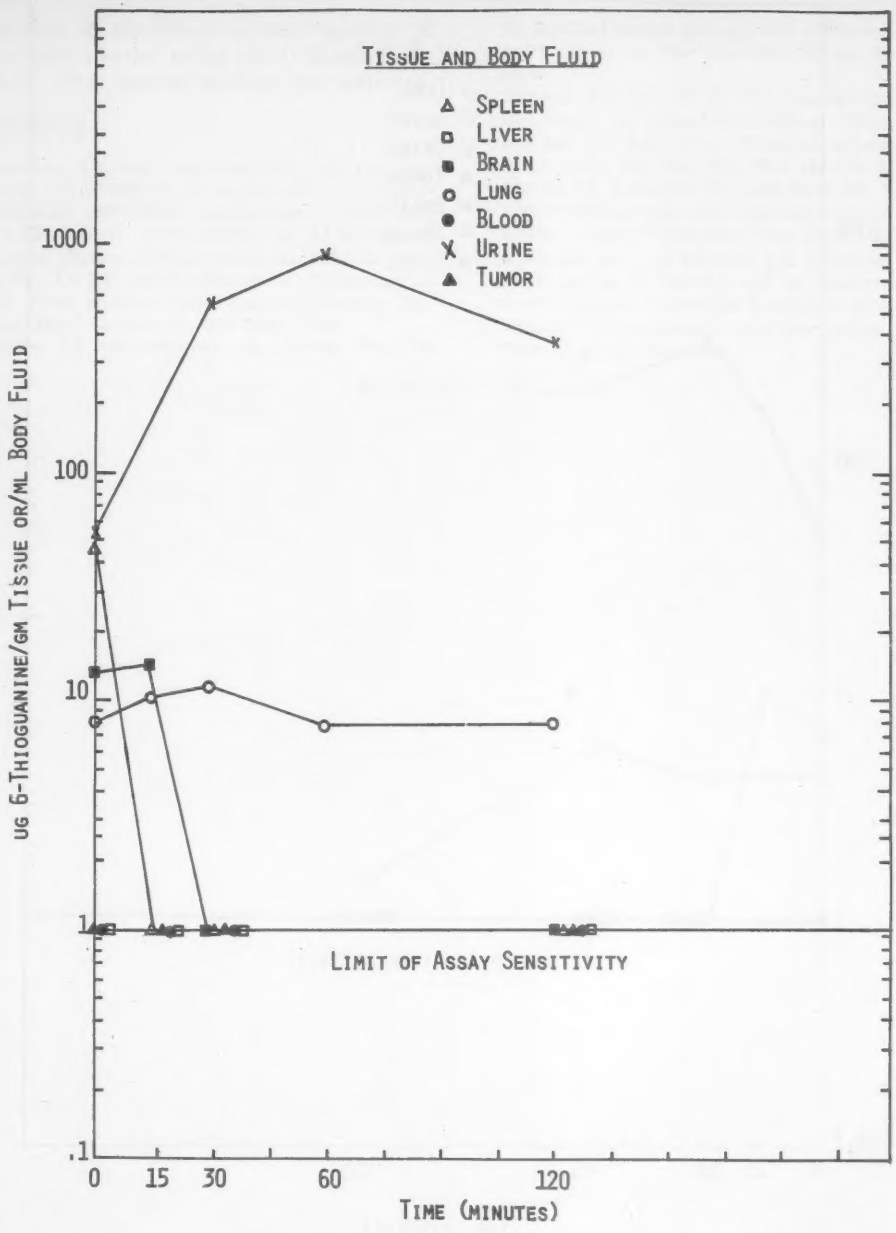

FIGURE 11.—Concentrations of 6-thioguanine (or cytotoxic equivalents) detected in tissues and body fluids of L1210-bearing mice by microbiological assay. Each point represents the average drug level of 5 mice. The assay microorganism was *E. coli* ATCC 9637/FPA. Mice were injected with a single ip dose (60 mg/kg) of drug.

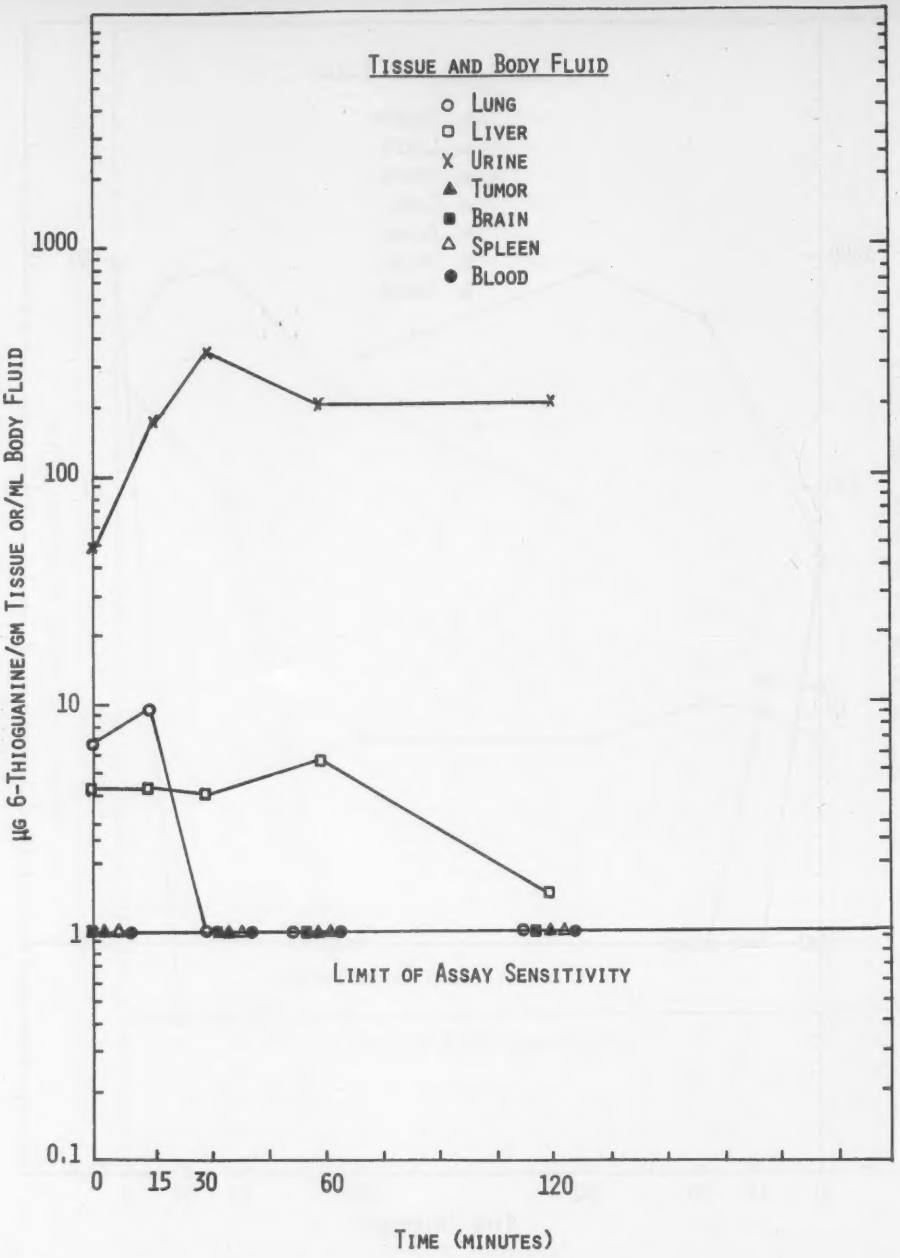

FIGURE 12.—Concentrations of 6-thioguanine (or cytotoxic equivalents) in combination with ara-C detected in tissues and body fluids of L1210-bearing mice by microbiological assay. Each point represents the average drug level of 5 mice. The assay microorganism was *E. coli* ATCC 9637/FPA. Mice were injected with a single ip dose of 60 mg/kg of 6-thioguanine and 100 mg/kg of ara-C simultaneously.

thioguanine is significant in our opinion. It appears that studies using ara-C in combination with other purine analogs are indicated.

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Value of Cytosine Arabinoside (NSC-63878) Plus 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) Therapy in Advanced Murine L1210 Leukemia and Enhancement of the Combination With Sequential Doses of Methotrexate (NSC-740) or 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU; NSC-409962) [1,2,3,4]

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SUMMARY

Treatment with daily doses of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) plus cytosine arabinoside (ara-C) (administered once every 3 hours for 24 hours every fourth day) was superior to treatment with either drug alone in mice with advanced L1210 leukemia. The optimal combination utilized doses of ara-C that are effective when given alone plus doses of NSC-45388 that are relatively ineffective when given alone. In five experiments, this combination elicited increases in survival time that were 39%–95% greater than those observed after optimal single-drug therapy. In the experimental model presented, methotrexate and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) given alone or combined following therapy with NSC-45388 plus ara-C provided increases in lifespan that were 55%–110% greater than those observed with the NSC-45388 plus ara-C combination. Bioassay studies showed that resistance to ara-C developed rapidly but that the recipients responded to NSC-45388 or methotrexate. These studies showed that the rapid development of resistance to ara-C apparently limited the efficacy of the ara-C plus NSC-45388 combination and indicated the usefulness of treatment with sequential doses of methotrexate and BCNU in enhancing the benefits of the combination.

[Cancer Chemother Rep 57:291–298, 1973]

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[1] Received Feb 20, 1973; revised May 29, 1973; accepted June 21, 1973.

[2] Supported in part by contract PH43-68-1283 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health.

[3] Cytosine arabinoside: CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C.

NSC-45388: CAS reg. No. 4342-03-4.

Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]-benzoyl]-.

BCNU: CAS reg. No. 154-93-8.

The compounds used in these studies were supplied by Drug Research and Development (DR&D), DCT, NCI.

[4] Presented in part at the 62nd annual meeting of the American Association for Cancer Research, Apr 1971, Chicago, Ill (12).

[5] Microbiological Associates, Inc., Bethesda, Md.

[6] DR&D, DCT, NCI, Bethesda, Md.

[7] The authors are grateful to Dr. Vaman S. Waravdekar, Dr. F. Lester Howsden, and Mr. A. E. Sirica for their interest and assistance in this study. The authors also acknowledge the valuable counsel of Dr. Richard D. Costlow in the preparation of this manuscript, and the technical assistance of Mr. E. Waynn Artis, Mr. David Elam, Mr. Carl Von Der Pool, and their staff in carrying out these experiments.

Reports of clinical therapy for acute leukemia using two or more antitumor agents indicate an advantage over treatment with single agents (1,2). Livingston and Carter indicated in their comprehensive review (3) that future approaches to the treatment of neoplasms will emphasize the use of rationally designed combination therapy.

The murine L1210 leukemia system has been shown to be a good indicator for active antitumor agents used singly in man; it has also been shown to be useful for demonstrating synergism between two drugs (4). In a related report from our laboratory (5) we showed that combination treatment with 5-fluorouracil[8] and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) was superior to treatment with either agent alone in the L1210 leukemia system. NSC-45388, used in our earlier L1210 combination studies, has been reported to be useful in the treatment of melanoma in man (6–8), and it is currently being used in clinical studies of combination treatment of other solid tumors (9) (Carter SK, personal communications).

The studies described in this report were designed to investigate the effect of the combination of NSC-45388 and cytosine arabinoside (ara-C) (an agent which is being used extensively in the clinic [3]) against L1210 leukemia. Biochemical studies (10) have shown that after ara-C is converted to the nucleoside triphosphate, it blocks DNA synthesis by inhibiting DNA polymerase. Inhibition of DNA synthesis (11) has been proposed as a possible mode of action of NSC-45388. It is conceivable that ara-C, which exerts its antitumor effect as a pyrimidine analog, and NSC-45388, which produces its effect as a purine analog, may produce a concurrent blockade in DNA production. The net effect of this combination of drugs might be antitumor activity greater than that achieved with either agent given alone.

In a preliminary report (12) from this laboratory, we described the enhanced therapeutic effect of ara-C plus NSC-45388. The investigations reported in this paper were conducted (*a*) to explore the development of resistance to ara-C as a factor limiting the therapeutic advantage of the combination of ara-C plus NSC-45388 and (*b*) to investigate the usefulness of treatment with methotrexate or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) after therapy with ara-C plus NSC-45388.

MATERIALS AND METHODS

The materials and methods used in this study were essentially the same as those described in previous reports from this laboratory (13,14). The ascitic form of L1210 leukemia used in these studies was carried by weekly transplantation of tumor cells into the peritoneal cavity of DBA/2 mice. Recipient BDF_1 (C57BL/6 female × DBA/2 male) or CDF_1 (BALB/c female × DBA/2 male) hybrid male mice that weighed 18–26 g were inoculated subcutaneously (sc) with 1×10^6 leukemia cells. Each treated group consisted of six to eight mice. Eight to 16 untreated animals were used in each experiment.

In addition to the treated and untreated leukemic mice used for survival studies, parallel groups of mice in three experiments received similar therapy. When local sc tumors were palpable, the spleens from these donors were transplanted into recipient mice of the same strain. These recipient animals, in turn, were challenged with the same drugs received by the donor animals in order to detect the development of drug resistance in one transfer generation under treatment.

An aqueous solution of ara-C was prepared fresh daily in pyrogen-free distilled water (pH adjusted to approximately 4.5 with 1N sodium hydroxide). NSC-45388 was prepared immediately before each injection (pH 6.0–6.4) in steroid suspending vehicle.[9]

The drugs were injected sc over a range of logarithmically spaced dose levels in the constant volume of 0.01 ml/g of body weight. The various treatment schedules used for the drugs

[8] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

[9] One ml of steroid suspending vehicle contained the following: sodium chloride, 9 mg; sodium carboxymethylcellulose, 5 mg; polysorbate-80, 0.004 ml; benzyl alcohol, 0.0009 ml; and water (to volume).

given singly, in combination, or sequentially are indicated in the tables and figures. In addition, various ratios of doses were used in the groups of mice receiving sequential therapy. The median survival time (MST) of the untreated mice is expressed as the percent increase in lifespan (% ILS) over the MST of the untreated leukemic mice.

RESULTS

The results of five experiments with ara-C and NSC-45388 given alone and in combination from Day 5 after tumor inoculation are summarized in table 1. While confirming the activity of each drug and the superiority of ara-C alone over NSC-45388 alone, the data demonstrated that the combination was markedly more effective than either drug alone. The maximum increase in survival time elicited by the combination over that elicited by maximally effective ara-C treatment was 50%–95% greater in four experiments and 39% greater in one experiment (table 1). In general, the most effective therapy was achieved by combining an optimal or near optimal dose of ara-C (5–8 mg/kg/injection) and a relatively low dose of NSC-45388 (39–180 mg/kg/injection) (ineffective when used alone). Data from these studies also indicate that the duration of treatment with ara-C administered alone did not appreciably influence the survival of the leukemic mice (table 1). This result may be related in part to the results from bioassay studies which suggest that resistance to ara-C develops rapidly (table 2).

In table 2, data from parallel bioassay studies performed with other groups of mice from experiment DL-666 (table 1) are summarized. Infiltrated spleens from leukemic donor mice treated on Days 5–13 with ara-C alone (treatment administered every 3 hours for 24 hours every fourth day [q3h/24h/q4d]) or on Days 5–29 with ara-C plus NSC-45388 (administered once daily) were transplanted into recipient mice of the same strain. The recipient mice were treated with ara-C, NSC-45388, or methotrexate alone and observed for survival. The lifespans observed indicated that the recipient

TABLE 1.—Treatment of advanced L1210 leukemia with ara-C plus NSC-45388*

Experiment No.	Control MST† (days)	Treatment schedule (days after tumor implantation)	Most effective dose (mg/kg/injection)‡			Increase in lifespan (%) at most effective dose		
			Ara-C	NSC-45388	Ara-C plus NSC-45388	Ara-C	NSC-45388	Ara-C plus NSC-45388
D–1699	9	5–32	23	300	8+108	200	55	250
D–1721	9	5–20	8	300	8+65	167	67	255
D–1889	9.5	5–17	8	300	8+65	131	36	226
		5–22	5	300	8+39	136	36	216
DL–651	9	5–13	14	300	5+108	144	50	183
		5–29	14	—	5+65	150	—	233
DL–666	10	5–13	8	500	8+180	110	35	165
		5–29	14	—	5+108	145	—	215

*10^6 ascites cells were implanted sc on Day 0. There were eight mice in each treated group and 16 mice in each untreated group.

† MST = median survival time of untreated controls.

‡ In each experiment, individual drug and combination treatments were given over a wide range of doses. For each therapy, the maximally effective dose (or combination) is shown. Ara-C was given sc every 3 hours for 24 hours on every fourth day (q3h/24h/q4d); this is the most effective schedule against early L1210 leukemia. In a recent paper from our laboratory (23) treatment of advanced L1210 leukemia with ara-C administered q3h/24h/q4d did not provide a therapeutic advantage over treatment with a single injection every second or fourth day. NSC-45388 was injected daily; this is an effective schedule for this agent which has not shown important schedule dependency when used against L1210 leukemia.

TABLE 2.—Effect of ara-C plus NSC-45388 therapy for donor mice on the response of recipient mice to ara-C, NSC-45388, or methotrexate treatment*

	Treatment of donor mice			Treatment and response of recipient mice				
Experiment No.	Drug	Dose (mg/kg/injection)	Treatment schedule	Drug	Optimal dose (mg/kg/injection)	Treatment schedule	MST† (days after tumor implantation)	Increase in lifespan (%)
DL–666a	Ara-C	5.0	q3h/24h/q4d,‡ Days 5–13	Ara-C	5–14	q3h/24h/q4d, Days 1–9	14	17
				NSC-45388	180	Daily, Days 1–17	20	67
				Methotrexate	2§	Daily, Days 1–17	23	92
				Untreated controls			12	
DL–666a	Ara-C	8.4	q3h/24h/q4d, Days 5–29	Ara-C	8.4	q3h/24h/q4d, Days 1–9	13	8
	+			NSC-45388	(180, 300)	Daily, Days 1–17	16	38
	NSC-45388	65.0	Daily, Days 5–29	Methotrexate	2§	Daily, Days 1–17	19	58
				Untreated controls			12	

* Leukemic infiltrated spleens from treated donor mice were transplanted sc into recipient mice of the same strain (BDF_1). There were six to eight mice in each treated group and eight to 16 mice in each untreated group.

† MST = median survival time.

‡ Treatment every 3 hours for 24 hours on every fourth day.

§ Only dose tested.

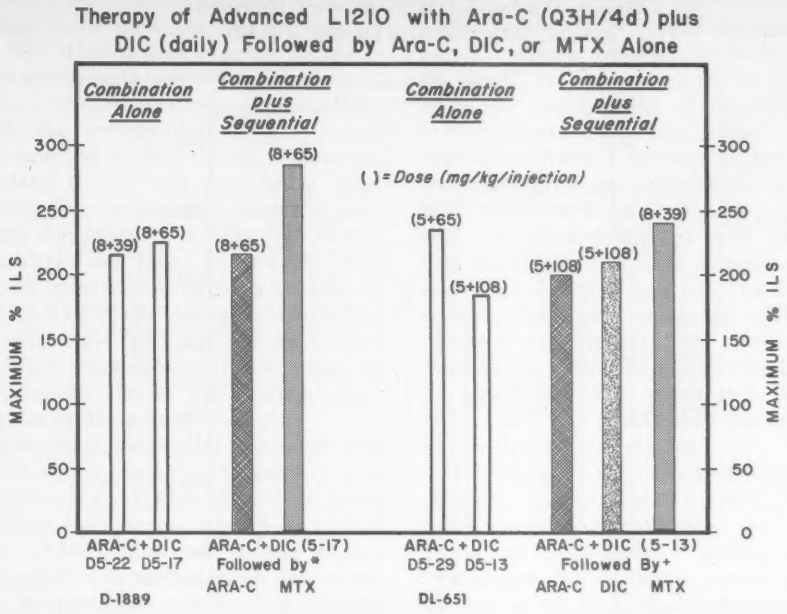

FIGURE 1.—Experiment D-1889. BDF$_1$ male mice (eight per treated group and 16 per untreated group) weighing 24–29 g at the beginning of the experiment were inoculated sc with 1×10^6 L1210 ascites cells. Treatment (sc) was started on Day 5 after tumor implantation. In the groups treated with the combination alone, ara-C was given q3h/24h/q4d and NSC-45388 (DIC) was given once daily on Days 5–17 or Days 5–22. In the groups that received sequential therapy, ara-C was given q3h/24h/q4d and NSC-45388 was given daily on Days 5–17.

*After ara-C plus NSC-45388 combination therapy (Days 5–17), animals were treated with either ara-C (300 mg/kg given once every second day) or methotrexate (MTX) (1.8 mg/kg given once daily) from Day 22 to death. The maximal response achieved with methotrexate given alone once daily on Days 5–22 was an ILS of 84%. Experiment DL-651. BDF$_1$ male mice weighing 20–30 g at the beginning of the experiment were inoculated sc with 1×10^6 L1210 ascites cells. Treatment (sc) was started on Day 5 after tumor inoculation. In the groups treated with the combination alone, ara-C was administered q3h/24h/q4d and NSC-45388 was administered once daily on Days 5–13 or Days 5–29. In the groups that received sequential therapy, ara-C was given q3h/24h/q4d and NSC-45388 was administered daily on Days 5–13.

†After combination therapy (Days 5–13) with ara-C plus NSC-45388, animals were treated with either ara-C (at a dose of 5 mg/kg/injection given q3h/24h/q4d), NSC-45388 (at a dose of 180 mg/kg given daily), or methotrexate (at a dose of 2.0 mg/kg given daily) on Days 17–29. Methotrexate given alone once daily on Days 5–29 gave a maximal response of 100% ILS. (*See* table 1 for the data obtained with ara-C and NSC-45388 given alone in experiments D-1889 and DL-651).

mice responded to NSC-45388 or methotrexate treatment but were resistant to ara-C treatment. The bioassay data from experiment DL-666 are representative of two replicate studies, D-1889 and DL-651 (table 1). These findings suggest that the rapid onset of resistance to ara-C may be one of the factors which limit the efficacy of the combination therapy (ara-C plus NSC-45388).

The experimental design and data from three experiments summarized in figures 1 and 2 illustrate the usefulness of sequential chemotherapy with methotrexate, BCNU, or methotrexate plus BCNU (agents which are active in mice with implants of an ara-C resistant variant of L1210 [15]) in enhancing the efficacy of combined treatment with ara-C and NSC-45388.

The data from experiment D-1889, presented in the left compartment of figure 1, indicate that sequential treatment with ara-C alone given every second day from Day 22 to death

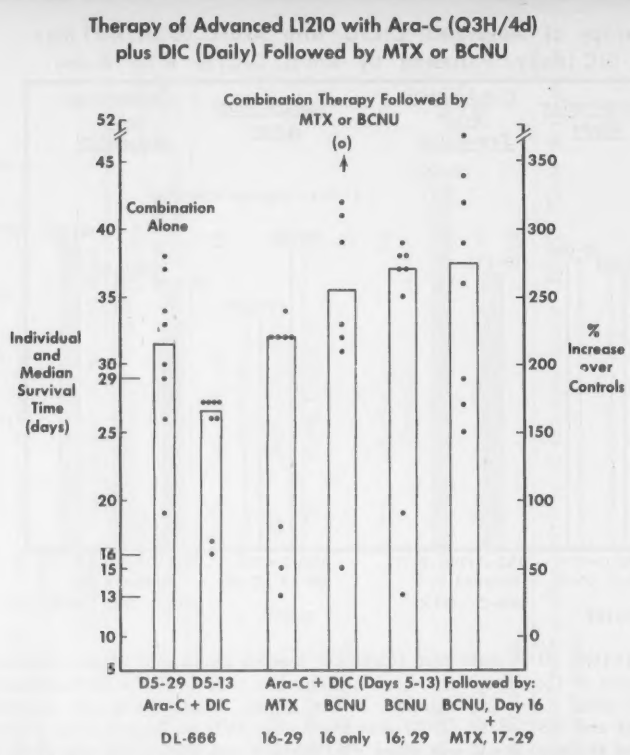

FIGURE 2.—Experiment DL-666. BDF_1 male mice (eight per treated group and 16 per untreated group) weighing 19–26 g at the beginning of the experiment were inoculated sc with 1×10^6 L1210 ascites cells. Treatment (sc) was started on Day 5 after tumor inoculation. In the groups that received the combination alone, ara-C was injected q3h/24h/q4d and NSC-45388 (DIC) was given once daily on Days 5–13 or Days 5–29. In the groups of mice that were to receive methotrexate (MTX) or BCNU after the combination, ara-C was given q3h/24h/q4d and NSC-45388 was given daily from Days 5–13. Following combination treatment with ara-C plus NSC-45388 on Days 5–13, treatment with either methotrexate (at a dose of 2.0 mg/kg given once daily on Days 16–29), BCNU (at a dose of 20 mg/kg given on Day 16 only), BCNU (at a dose of 20 mg/kg given on Days 16 and 29 only), or BCNU (at a dose of 20 mg/kg given on Day 16) plus methotrexate (at a dose of 2.0 mg/kg given on Days 17–29) was administered. Methotrexate given alone once daily on Days 5–29 produced a maximal effect of 60% ILS. BCNU given alone on Day 5 only produced a >500% ILS and treatment with this agent on Days 5 and 18 only achieved a maximal antileukemic effect of >370% ILS. (Refer to table 1 for the data for ara-C and NSC-45388 given alone.)

after 4 days of respite from combination therapy with ara-C plus NSC-45388 on Days 5–17 did not increase survival. In the same experiment, daily sequential treatment with methotrexate increased survival by 58% over that produced with the combination of ara-C plus NSC-45388 (fig 1).

In the second experiment (DL-651, fig 1), sequential therapy with ara-C or NSC-45388 increased survival by 17% or 28%, respectively, over the combination of ara-C plus NSC-45388 given on Days 5–13; in contrast, sequential daily therapy with methotrexate from Days 17–29 enhanced survival by 56%. In this experiment sequential therapy with methotrexate was not superior to treatment with the combination of ara-C plus NSC-45388 alone administered on Days 5–29.

The data from experiment DL-666 presented in figure 2 indicate that treatment with BCNU (given either on Day 16 or on Days 16 and 29 of the experiment following treat-

ment on Days 5–13 with the combination of ara-C plus NSC-45388) was superior to treatment with methotrexate and extended survival by 95% and 105%, respectively, over that observed with the combination of ara-C and NSC-45388 injected on Days 5–13. Sequential treatment with BCNU was also better than treatment with the combination when the combination was continued until Day 29. BCNU given sequentially on Days 16 and 29 after termination of combination therapy on Day 13 produced an ILS of 270%, whereas combination treatment with ara-C plus NSC-45388 elicited an ILS of 165% when treatment was given on Days 5–13, and an ILS of 215% when treatment was given on Days 5–29.

In one experiment, sequential treatment with methotrexate was superior to treatment with the combination of ara-C plus NSC-45388 given either on Days 5–17 or on Days 5–22 (fig 1, experiment D-1889). However, in two experiments, treatment with methotrexate was clearly superior to treatment with the combination on Days 5–13 but was comparable to treatment with the combination on Days 5–29 (fig 1, experiment DL-651; fig 2). Sequential therapy with BCNU or methotrexate plus BCNU was superior to maximally effective combination therapy administered alone on Days 5–29 (fig 2). The experimental model presented here is a tool for investigating the ability of sequential therapy with a variety of agents to enhance the benefits achieved with combination therapy.

DISCUSSION

The current observation that an enhanced therapeutic effect occurred in the L1210 leukemia system when optimal doses of ara-C were combined with suboptimal doses of NSC-45388 is similar to our reported observation (5) of improved therapeutic effect with 5-FU plus NSC-45388 when optimal doses of 5-FU were given with relatively ineffective doses of NSC-45388. In both studies, the additive toxicity to the host appeared to limit the additive antileukemic effect of these agents.

Since resistance to treatment with ara-C develops rapidly in mice with L1210 leukemia (12,15,16), resistance to ara-C may be a factor in the limited efficacy of this combination. It is of particular interest therefore that methotrexate, BCNU, and methotrexate plus BCNU (agents reported to be active against an ara-C resistant variant of L1210 [15]) enhanced the benefits of combination treatment with ara-C plus NSC-45388. Even though resistance to ara-C develops rapidly, prolonged treatment with the combination of ara-C and NSC-45388 on Days 5–29 gave a 50% greater ILS than limited therapy with this combination on Days 5–13 (*see* experiments DL-651 and DL-666, figs 1 and 2). The data in table 2 show that mice implanted with the ara-C resistant variant of L1210 are responsive to NSC-45388. This may account for the increased survival time with prolonged therapy with NSC-45388 and ara-C even though resistance to ara-C was demonstrated.

The current studies were prompted by continued clinical interest in ara-C, which blocks DNA synthesis by inhibiting DNA polymerase (10), and NSC-45388, a structural analog of precursors for de novo purine biosynthesis, as well as the hope that enhanced therapeutic effect would result with combination therapy by concurrent blockade of DNA biosynthesis. Although the antitumor mechanism of action of NSC-45388 is unknown, Saunders and Schultz (11) reported that high levels of NSC-45388 inhibited DNA synthesis in bacteria. Skibba et al (17–19) studied the metabolism of NSC-45388 and reported that *N*-demethylation appears to represent a major metabolic pathway for this agent in man and rats. The precise mechanism of the enhanced therapeutic effect produced by the combination of ara-C and NSC-45388 described here must still be elucidated.

We hope that the experimental model presented here will be useful to the chemotherapist in the selection of groups of agents for clinical evaluation. The therapeutic advantage of combination and sequential chemotherapy in the management of clinical leukemia has been summarized by Frei and Freireich (1), Henderson (2), and Sellei et al (20). Frei et al (21), Hiatt (22), and other clinical chemotherapists have pointed out the usefulness of combination chemotherapy in delaying the development of drug resistance. Other clinical implications of these studies are related to the potential ability of drugs given sequentially to inhibit

tumor growth before toxicity to the host occurs.

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Protection With *N*-Acetyl-L-cysteine (NSC-111180) Against Isophosphamide (NSC-109724) Toxicity and Enhancement of Therapeutic Effect in Early Murine L1210 Leukemia [1,2,3,4]

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SUMMARY

Treatment with *N*-acetyl-L-cysteine protected mice with L1210 leukemia against the lethal toxicity of isophosphamide resulting in an increase in therapeutic efficacy. A single dose of 500 mg/kg of isophosphamide was lethal to leukemic and nonleukemic mice. When *N*-acetyl-L-cysteine was given prior to the administration of the 500-mg/kg dose of isophosphamide on Day 1 after tumor implantation, the combination was consistently tolerated. In each of five experiments, the combination of *N*-acetyl-L-cysteine plus isophosphamide elicited an increase in lifespan that was two to three times greater than that elicited by treatment with the optimal dose (300 mg/kg) of isophosphamide alone. Both protection against toxicity to the host and enhanced antitumor effect were observed when *N*-acetyl-L-cysteine was given simultaneously with, 1 hour prior to, or 10 minutes after isophosphamide. Histologic studies showed that isophosphamide-induced hemorrhage in the bladder was minimized in mice given *N*-acetyl-L-cysteine.

[Cancer Chemother Rep 57:299–304, 1973]

The hematologic toxicity of cyclophosphamide [8] is being partially controlled in the clinic by platelet and white blood cell (wbc) transfusions; however, toxicity to the bladder has been reported to be a dose-limiting factor in the clinical use of higher therapeutic doses (1,2). In experimental models designed to study this problem, Primack (3) reported that irrigation of the bladders of monkeys and dogs with acetylcysteine protected the animals against cyclophosphamide-induced cystitis. Isophosphamide, a position isomer of cyclophosphamide with demonstrable antitumor activity in animals (4), is being evaluated clinically,

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[2] Supported in part by contract PH43-68-1283 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health.

[3] *N*-Acetyl-L-cysteine: CAS reg. No. 616-91-1; acetylcysteine.

Isophosphamide: CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)-amino]tetrahydro-, 2-oxide.

The compounds used in these studies were supplied by Drug Research and Development (DR&D), DCT, NCI.

[4] Presented in part at the 63rd annual meeting of the American Association for Cancer Research, May 1972, Boston, Mass. [Kline I, Gang M, and Venditti JM. Protection with *N*-acetylcysteine (NAC) against isophosphamide (Isoph, NSC-109724) host toxicity and enhancement of therapy in murine leukemia L1210. Proc Am Assoc Cancer Res 13:29, 1972.]

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[6] Drug Evaluation Branch, DR&D, DCT, NCI, Bethesda, Md.

[7] The authors wish to acknowledge the valuable technical assistance of Mr. E. Waynn Artis, Mr. David Elam, Mr. C. Von Der Pool, and their staff.

[8] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

and the preliminary clinical reports indicate that the drug induces severe cystitis (Carter SK, personal communication). The observations of Primack (3) and earlier reports reviewed by Connors (5) indicating that sulfhydryl-containing compounds protect against the toxicity of alkylating agents prompted the current studies which are designed to determine whether *N*-acetyl-L-cysteine protects mice against isophosphamide toxicity and enhances the therapeutic effect of the drug against murine L1210 leukemia.

MATERIALS AND METHODS

The materials and methods used in this study were essentially the same as those described in previous reports from this laboratory (6,7). The ascitic form of L1210 leukemia used in these studies was carried by weekly transplantation of leukemic cells into the peritoneal cavity of DBA/2 mice. The experimental inocula of tumor cells were implanted either intraperitoneally (ip) (1×10^5 cells/mouse) or subcutaneously (sc) (1×10^6 cells/mouse) in BDF_1 (C57BL/6 female × DBA/2 male) hybrid mice weighing between 18 and 28 g at the beginning of the experiments.

An aqueous solution of isophosphamide was prepared fresh daily in pyrogen-free distilled water (pH 4.5–7.0). *N*-Acetyl-L-cysteine was dissolved fresh daily in physiologic saline (pH 6.8–7.0).

The drugs were given alone or in sequential combination over a wide range of logarithmically spaced dose levels in a constant volume of 0.01 ml/g of body weight. The various schedules of treatment are indicated in the tables, figure, and text.

The parameters of antitumor efficacy were (*a*) increase in survival time and (*b*) number of long-term survivors. The median survival time (MST) of the treated mice is expressed as percentage increase in lifespan (% ILS) over the MST of the untreated leukemic mice. In certain experiments, parallel groups of non-tumor-bearing mice received the same therapy as the leukemic mice in the same experiment.

RESULTS

Figure 1 illustrates the enhanced antileukemic (L1210) therapeutic effect resulting from the ability of *N*-acetyl-L-cysteine (an agent without antitumor activity) to reduce the toxicity of isophosphamide to the host without reducing toxicity to the tumor. For example, the optimal therapeutic response for a single treatment with isophosphamide alone was achieved with a dose of 300 mg/kg. This dose was nonlethal to normal mice and produced an MST of 23 days in the leukemic mice. A single treatment with 500 mg/kg of isophosphamide produced four deaths among six non-tumor-bearing mice and five very early deaths and only one 60-day survivor among eight leukemic mice. However, all of the six non-tumor-bearing mice treated with *N*-acetyl-L-cysteine tolerated the 500-mg/kg dose of isophosphamide, and six of eight leukemic mice treated with *N*-acetyl-L-cysteine survived for 60 days.

The five experiments summarized in table 1 illustrate the reproducibility of the effect of *N*-acetyl-L-cysteine on the reduction of isophosphamide toxicity resulting in enhanced isophosphamide antileukemic activity. For example, a single ip injection of 500 or 833 mg/kg of isophosphamide alone was consistently toxic to leukemic or nonleukemic mice. The optimal dose for isophosphamide alone (300 mg/kg) provided increases in MST ranging from 60% to 161%. When isophosphamide treatment was preceded by *N*-acetyl-L-cysteine treatment, the combination of 750–2000 mg/kg of *N*-acetyl-L-cysteine plus 500 mg/kg of isophosphamide provided increases in lifespan that were two or three times greater than those observed with the optimal dose of isophosphamide alone.

The experiments summarized in table 2 show the effect of variation of the interval between administration of *N*-acetyl-L-cysteine and isophosphamide on toxicity to the host and therapeutic efficacy. Whether *N*-acetyl-L-cysteine was given simultaneously with, 1 hour prior to, or 10 minutes after isophosphamide at a dose of 500 mg/kg, the results were comparable.

In experiment 5 (table 1) sufficient numbers of leukemic or nonleukemic mice were included in each of the groups to collect survival data and to supply tissues for macroscopic and microscopic bladder examinations. At various in-

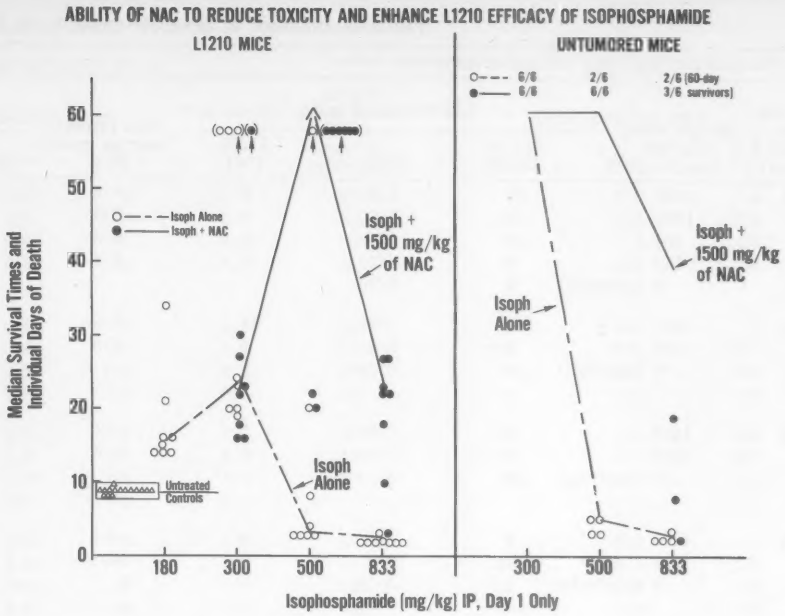

FIGURE 1.—BDF_1 male mice (eight leukemic and six nonleukemic mice per treated group and 16 untreated leukemic controls) weighing 18–25 g at the beginning of the experiment were inoculated ip with 1×10^5 L1210 ascites cells. Treatment (ip) with each drug alone or with the combination of *N*-acetyl-L-cysteine (NAC) plus isophosphamide (Isoph) was given once (on Day 1 after tumor inoculation). When *N*-acetyl-L-cysteine was given in the combination treatment, it was administered approximately 10 minutes before the isophosphamide. Treatment with *N*-acetyl-L-cysteine alone at a dose of 1500 mg/kg produced an MST of 9 days in the leukemic mice. The number of 60-day survivors in each group of eight leukemic mice is shown within parentheses.

tervals after treatment, untreated and treated animals were killed. Their bladders were removed, and the tissues were examined and then fixed in 10% formalin prior to hematoxylin and eosin staining for microscopic evaluation.

Confluent areas of hemorrhage were noted in bladders dissected (on Day 7) from leukemic and nonleukemic mice treated with a single injection of isophosphamide alone (500 mg/kg) on Day 1 after tumor implantation. Histologic examination revealed that these same tissues had hemorrhagic abnormalities. In contrast, minimal signs of hemorrhage were detected in the bladders of mice treated with *N*-acetyl-L-cysteine (1500 mg/kg) on Day 1 of the experiment prior to the administration of isophosphamide (500 mg/kg). This observation correlates with the data indicating increases in survival time in table 1.

Additional studies were done to explore the influence of *N*-acetyl-L-cysteine on isophosphamide in mice with the advanced stage of L1210 leukemia. Treatment with *N*-acetyl-L-cysteine, whether given simultaneously with, before, or after isophosphamide, alleviated the toxicity of a single injection of isophosphamide given on Day 5 after tumor implantation but did not produce a therapeutic effect better than that observed with isophosphamide given alone. Responses to daily or weekly treatments with *N*-acetyl-L-cysteine plus isophosphamide were not superior to the responses seen with isophosphamide given alone to mice with advanced L1210 leukemia.

TABLE 1.—Influence of *N*-acetyl-L-cysteine on the toxicity

Experiment No.	Observation period (days)	*N*-Acetyl-L-cysteine alone: Single dose† (mg/kg/injection)	L1210-bearing mice: MST‡	S/T§	ILS¶ (%)	Non-tumor-bearing mice, S/T	Single dose (mg/kg/injection)
1	60	2000	9	0/8	0	7/8	833
		1000	9	0/8	0	8/8	500
		500	9	0/8	0	8/8	300
		250	9	0/8	0	8/8	180
		0 (controls)	9	0/16			
2	60	1500	10	0/8	11	8/8	833
		1000	9	0/8	0	8/8	500
		0 (controls)	9	0/16			300
							180
3	60	1500	9	0/8	0	6/6	833
		1000	9	0/8	0	6/6	500
		0 (controls)	9	0/16			300
							180
4	45	1500	9	0/8	0	6/6	833
		1000	9	0/8	0	6/6	500
		0 (controls)	9.5	0/16			300
							180
5	60	1500	10	0/27	0	26/26	833
		750	10	0/30	0	30/30	500
		375	9	0/10	0	10/10	300
		0 (controls)	10	0/33			180

*Male BDF$_1$ mice (18–28 g) were inoculated ip with 1×10^5 ascites cells.

†Ip treatment (started on Day 1 after tumor implantation) was given over a wide range of logarithmically spaced doses.

‡MST = median survival time.

§S/T = No. of survivors/total No. of treated animals.

¶Percent increase in median lifespan over that of control animals.

**N-Acetyl-L-cysteine was injected 10 minutes prior to isophosphamide. The dose of *N*-acetyl-L-cysteine given in optimal combination with isophosphamide in each experiment is shown below.

Experiment No.	*N*-Acetyl-L-cysteine dose (mg/kg/injection)
1	2000
2	750
3	1500
4	1500
5	1500

DISCUSSION

Data from the survival studies presented in this paper show that pretreatment with *N*-acetyl-L-cysteine protected the host against isophosphamide toxicity but did not reduce toxicity to the tumor. These observations were supported by macroscopic and microscopic observations which showed that *N*-acetyl-L-cysteine minimized the localized hemorrhage induced in the bladders by isophosphamide. In addition, the administration of *N*-acetyl-L-cysteine elicited a therapeutic effect that was superior to the effect provided by isophosphamide alone. In this respect, *N*-acetyl-L-cysteine enhanced the antileukemic effect of higher, more effective doses of isophosphamide without reducing the therapeutic effect of lower doses of the drug.

and activity against L1210 leukemia of isophosphamide*

Isophosphamide alone				*N*-Acetyl-L-cysteine plus isophosphamide**			
L1210-bearing mice			Non-tumor-bearing mice S/T	Single isophosphamide dose (mg/kg/injection)	L1210-bearing mice			Non-tumor-bearing mice S/T
MST (days)	S/T	ILS (%)			MST (days)	S/T	ILS (%)	
2	0/8	0	0/8	833	7.5	0/8	0	2/8
7.5	1/8	0	4/8	500	24.5	0/8	172	7/8
17.5	0/8	94	8/8	300	16.5	1/8	83	8/8
14	0/8	55	8/8	180	14	0/8	55	8/8
2	0/8	0	0/8	833	2	2/8	0	0/8
3	0/8	0	0/8	500	>60	6/8	>567	5/8
20.5	0/8	128	8/8	300	35.5	2/8	295	5/8
16	0/8	78	8/8	180	16	0/8	78	8/8
2	0/8	0	2/6	833	22	0/8	144	3/6
3.5	1/8	0	2/6	500	>60	6/8	>567	6/6
23.5	3/8	161	6/6	300	22.5	1/8	150	6/6
15.5	0/8	72	6/6					
2.5	0/8	0	1/6	833	8	0/8	0	3/6
14	1/8	47	5/6	500	26.5	3/8	179	6/6
18	2/8	89	6/6	300	18	1/8	89	6/6
16	0/8	68	6/6	180	15.5	0/8	63	6/6
2	0/25	0	0/23	833	4	2/21	0	4/20
3.5	3/20	0	2/20	500	24	2/24	140	22/23
16	2/27	60	22/28	300	18	2/30	80	25/29
14.5	0/30	45	10/10	180	14	0/30	40	9/10

In a review, Bacq (8) described the protection against X-irradiation afforded by various chemicals, including compounds containing sulfhydryl groups. The reduction of the toxicity of alkylating agents by sulfhydryl-containing chemicals has been noted by Connors (5). The exact mechanism for the protection of the host against drug toxicity and the enhanced therapeutic response observed in the current experiments must still be elucidated, but one possibility is that the protective action of *N*-acetyl-L-cysteine is similar to that described for sulfhydryl-containing compounds in experimental models of treatment with other alkylating agents.

The use of platelet and wbc transfusions and "Life Island" isolators has aided the clinician in the supportive care of patients, and it

TABLE 2.—Influence of interval between administration of *N*-acetyl-L-cysteine and administration of isophosphamide on toxicity and activity against L1210 leukemia*

Interval (min) between *N*-acetyl-L-cysteine dose (1500 mg/kg) and isophosphamide dose (at time 0)	Single isophosphamide dose†								
	833 mg/kg			500 mg/kg			300 mg/kg		
	L1210-bearing mice		Non-tumor-bearing mice	L1210-bearing mice		Non-tumor-bearing mice	L1210-bearing mice		Non-tumor-bearing mice
	MST‡ (days)	S/T§	S/T	MST (days)	S/T	S/T	MST (days)	S/T	S/T
Isophosphamide alone	2	0/8	2/6	3.5	0/8	2/6	23.5	3/8	5/6
−10	22	0/8	3/6	> 60	6/8	6/6	22.5	1/8	6/6
−30	2	0/8	1/6	> 60	5/8	6/6	22.5	3/8	6/6
−60	6.5	2/8	4/6	> 60	6/8	3/6	23.5	2/8	6/6
Simultaneous administration	7	0/8	2/6	> 60	8/8	4/6	24.5	3/8	6/6
+10	9	1/8	1/6	> 60	5/8	5/6	23	1/8	6/6

* Male BDF_1 mice (18–25 g) were inoculated ip with 1×10^6 ascites cells.

† Ip treatment (started on Day 1 after tumor implantation) was given over a wide range of logarithmically spaced doses.

‡ MST = median survival time. The MST of the untreated control was 9 days.

§ S/T = No. of 60-day survivors/total No. of treated animals.

also permits the optimal use of cytotoxic agents in cancer chemotherapy (9). The use of *N*-acetyl-L-cysteine for protection against toxicity to the host may be a possible adjunct to the aggressive use of alkylating agents in the clinic.

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Effect of Streptonigrin (NSC-45383) and Analogs on Oncornavirus Replication and DNA Polymerase Activity [1,2]

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SUMMARY

The antibiotic streptonigrin was shown to be active in nanogram quantities against Moloney leukemia virus replication in vitro. In microgram quantities it was also effective against the Rauscher leukemia virus in vivo, and it markedly inhibited the DNA polymerase of a C-type RNA virus (avian myeloblastosis virus). Two analogs of streptonigrin (isopropylideneazastreptonigrin and the methyl ester of streptonigrin) were also tested. Minor to major alterations in the parent structure resulted in varied rates of activity.

[Cancer Chemother Rep 57:305–309, 1973]

Of the several approaches considered for the treatment of oncornavirus infection, chemotherapy has been investigated most extensively. The ultimate goal in viral chemotherapy is to synthesize a drug which has a specific deleterious effect on the virus itself and/or on viral replication.

Following the discovery of the RNA-dependent DNA polymerase (reverse transcriptase) in oncogenic RNA virus by Temin and Mizutani (1) and by Baltimore (2), intensive studies ensued on the distribution of this enzyme in oncogenic viruses and in different tumor cells and on the possibility of selectively inhibiting the reverse transcriptase by different agents (3–9). Calvin et al (4) reported on the different inhibitory effects of rifampicin [6] and two of its derivatives on Moloney sarcoma virus replication. Gurgo et al (6) showed that other derivatives of rifampicin were effective.

Streptonigrin, an antibiotic derived from the crude culture of *Streptomyces flocculus* (10), was characterized physically and chemically by Rao et al (11). Recently we reported marked (in vitro) inhibition of viral replication with this antibiotic against murine leukemia viruses (Moloney leukemia virus, Rauscher leukemia virus, and AKR leukemia virus) and murine sarcoma virus (MSV[M]). Excellent activity was also achieved in vivo against Rauscher leukemia virus and MSV(M). Streptonigrin was also found to inhibit avian myeloblastosis reverse transcriptase (5).

The objective of this study was to assess whether minor structural modifications of the parent antibiotic streptonigrin would alter its in vitro and in vivo antiviral activity.

[1] Received Mar 6, 1973; revised May 21, 1973; accepted May 24, 1973.

[2] Streptonigrin: CAS reg. No. 3930-19-6; picolinic acid, 5-amino-6-(7-amino-5,8-dihydro-6-methoxy-5,8-dioxo-2-quinolyl)-4-(2-hydroxy-3,4-dimethoxyphenyl)-3-methyl-. Streptonigrin, isopropylideneazastreptonigrin, and the methyl ester of streptonigrin were supplied by the Drug Development Branch (DDB), Drug Research and Development (DR&D), Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

[3] Viral Biology Branch, Division of Cancer Cause and Prevention, NCI, Bethesda, Md.

[4] DDB, DR&D, DCT, NCI, Bethesda, Md.

[5] Microbiological Associates, Inc., Walkersville, Md.

[6] Rifampicin: NSC-113926; CAS reg. No. 13292-46-1; 2,7-(epoxypentadeca[1,11,13]trienimino)naphtho[2,1-*b*]-furan-1,11(2*H*)-dione, 5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[*N*-(4-methyl-1-piperazinyl)formimidoyl]-, 21-acetate; rifampin.

MATERIALS AND METHODS

Antibiotics

Streptonigrin and the two structural analogs tested in this study are shown in figure 1. The methyl ester[7] represents only a minor change in the "C" ring where a nonpolar methyl group is introduced. Major alterations from the parent compound were made in the aza-isopropylidene analog.[8] A hydroxyl radical and aza-isopropylidene were introduced in the "A" ring and two oxygens were lost.

The antibiotics were either made up in RPMI-1640 medium in stock concentrations of 0.1 mg/ml for tissue culture studies or prepared in sterile phosphate-buffered saline (pH 7.0) for the in vivo studies.

In Vivo Assay

Murine leukemia virus (Rauscher) was assayed according to the method previously reported (12). The treatment schedule and doses used in this study were selected from a dose-response study conducted with each antibiotic in 2–3-month-old male BALB/c mice. Mice tolerated this schedule with little weight loss. Higher doses (greater than 150 μg/kg) were not tolerated well, and treatment led to marked losses in body weight.

In Vitro Assay

The XC cell assay was used to measure leukemia virus titers according to the method of Rowe et al (13). Essentially, plates were seeded with mouse embryo fibroblast (MEF) cells and incubated for 24 hours. Prior to the addition of virus and appropriate concentration of antibiotic the cells were treated with diethylaminoethyl (DEAE) dextran. After the cells were infected cultures were fed every 2 days. Five days after infection the MEF cultures were treated with ultraviolet irradiation and

STREPTONIGRIN AND STRUCTURAL ANALOGS

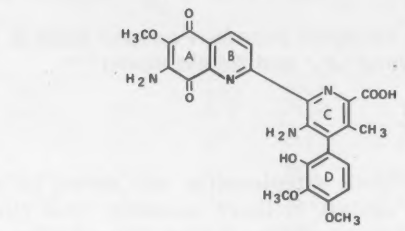

FIGURE 1.—Streptonigrin and two structural analogs.

overlaid with XC cells. Five days later the cells were fixed with methanol; subsequently they were stained with 35% hematoxylin, air dried, and read for syncytia. At concentrations of less than 15 ng/ml of streptonigrin, no growth-inhibitory effect on MEF cells was noted. Concentrations of more than 3 μg/ml of the methyl ester and aza-isopropylidene analogs resulted in a 10% growth inhibition.

DNA Polymerase Assay

For the determination of the effect of streptonigrin and its two analogs on avian myeloblastosis virus RNA-dependent DNA polymerase, the enzyme was isolated and purified essentially, as described by Kacian et al (14), by chromatography DEAE cellulose and phosphocellulose. The activity of the purified enzyme is completely dependent on an exogenous template.

The assay mixture (0.1 ml) contained the following: tris-HCl (pH 8.3), 5 μmol; $MgCl_2$, 0.6 μmol; dithiothreitol, 0.04 μmol; KCl, 10

[7] Streptonigrin, methyl ester: NSC-45384; picolinic acid, 5-amino-6-(7-amino-5,8-dihydro-6-methoxy-2-quinolyl)-4-(2-hydroxy-3,4-dimethoxyphenyl)-3-methyl-, methyl ester.

[8] Isopropylideneazastreptonigrin: NSC-62709; CAS reg. No. 15964-31-5; picolinic acid, 5-amino-4-(2-hydroxy-3,4-dimethoxyphenyl)-6-(5-hydroxy-4-methoxy-2,2-dimethyl-2*H*-imidazo-[4,5-*h*]quinolin-8-yl)-3-methyl-; IPAS.

μmol; adenosine triphosphate, 0.02 μmol; tritiated thymidine triphosphate, 4000 counts/minute/pmol; poly rA oligo dT, 2 μg; and avian myeloblastosis virus DNA polymerase, phosphocellulose fraction, 0.5 μg. Samples were incubated for 30 minutes at 37 C, and the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The precipitate was collected on glass fiber filters (Whatman GF/C). Radioactivity was measured in a 10-ml Aquasol spectrofluor scintillator. Streptonigrin and its analogs were dissolved in 0.5% formamide to a final concentration of 0.2 mg/ml. Formamide at this concentration (0.5%) was not found to inhibit the enzyme.

RESULTS

The results of in vitro testing of streptonigrin and the two analogs are shown in table 1. The parent compound demonstrated excellent in vitro inhibition of leukemia virus replication: 5 ng of streptonigrin resulted in a 100% inhibition. At the same concentration the two analogs were completely inactive. When the concentration of the methyl ester was increased, viral inhibitory activity was attained (at 250 ng); nearly 100% inhibition was achieved with 2500 ng (2.5 μg). It is of interest that a 500-fold increase in the concentration of the methyl ester was required to attain the inhibition achieved with 5 ng of the parent compound. The aza-isopropylidene analog was completely inactive at any concentration tested.

Results of in vivo testing of streptonigrin and the two analogs are shown in table 2. The parent streptonigrin compound was more effective than either of the two analogs. Treatment with streptonigrin resulted in a marked decrease in virus titer, an increase in survival time, and a significant number of long-term survivors. The response to the methyl ester analog was considered marginal. At the 100-μg/kg dose level, a 1-log reduction in virus was attained, as well as a protracted survival time and long-term survival. Again, similar to the in vitro results, the aza-isopropylidene analog was inactive.

The degree of activity of the three compounds in the inhibition of the DNA polymerase of avian myeloblastosis RNA tumor virus (fig 2) was similar to that seen in the in vitro and in vivo tests. The inhibition of the viral DNA polymerase was progressive with increasing concentration. At the 80-μg concentration, streptonigrin inhibited viral DNA polymerase activity by 50%, the methyl ester by 15%, and the aza-isopropylidene analog by only a negligible amount.

DISCUSSION

Although only two analogs of streptonigrin were tested, it is evident that minor changes in the polar group of the "C" ring affected the inhibitory activity of the compounds, and major alteration of the "A" ring resulted in abolition of the activity. The changes in activity were apparent in all three test systems.

TABLE 1.—Effects of streptonigrin and analogs on XC syncytial formation by murine leukemia virus (Moloney)

Concentration (ng/ml)	Inhibition (%) by—		
	Streptonigrin parent*	Streptonigrin, methyl ester	Streptonigrin, aza-, isopropylidene
2500	NT†	98	0
1250	NT	75	0
625	NT	57	0
250	NT	47	0
5.0	100	0	0
1.0	92	0	0
0.50	80	0	0
0.25	29	0	0

*Based on control values.
† NT = not tested.

TABLE 2.—In vivo effect of streptonigrin and analogs on murine leukemia virus (Rauscher) infection of mice*

Drug	Concentration (μg/kg)	Virus recovery † (logs)	Median survival time (days)	Range of deaths (days)	Survivors (%)
Control	None	5.0	56	40–95	0
Streptonigrin parent	100	1.0	124	51–>135	40
	50	3.0	61	42–>135	10
Streptonigrin, methyl ester	100	4.0	68	45–>135	10
	50	4.5	58	42–>135	10
Streptonigrin, aza-, isopropylidene	100	5.0	60	39–120	0
	50	5.0	50	22–75	0

*BALB/c mice (15 per group) were infected with 0.2 ml of a 10^{-2} dilution of murine leukemia virus (Rauscher). Seven days after infection, when mice were viremic, treatment was started with one treatment per day daily for a total of seven treatments. Drug was administered subcutaneously in the axillary area. One day after the last treatment five mice from each group were killed for virus bioassay (12). The remaining ten mice were held for determination of survival time.

†Results for bioassay 1 day after the last treatment.

The in vitro inhibitory activity of streptonigrin on syncytial formation by the Moloney leukemia virus was not considered to be due to a reduced number of cellular divisions since at the 5-ng/ml concentration the cell number was similar to that in control cultures. Calvin et al (4) reported similar marked inhibition of focus formation by murine sarcoma virus by exposure to 3 μg/ml of DMB-rifampicin without reduction in cell replication. It remains to be determined whether the activities associated with streptonigrin reflect a basic mechanism of action in that inhibition of the DNA polymerase per se can prevent infection of cells (demonstrated in vitro) and, therefore, viral leukemogenesis (demonstrated in vivo). Ting et al (15) observed a direct correlation between inhibition of viral DNA polymerase and inhibition of murine sarcoma virus focus formation as well as infectivity of Rauscher leukemia virus by different classes of rifamycin SV* derivatives, indicating that DNA polymerase is necessary for cell transformation. Alternatively, streptonigrin could also act on translation of the viral RNA message, preventing formation of the enzyme. The different order of magnitude of effective doses for infection and enzyme inhibition may reflect such a dual mechanism of action of streptonigrin.

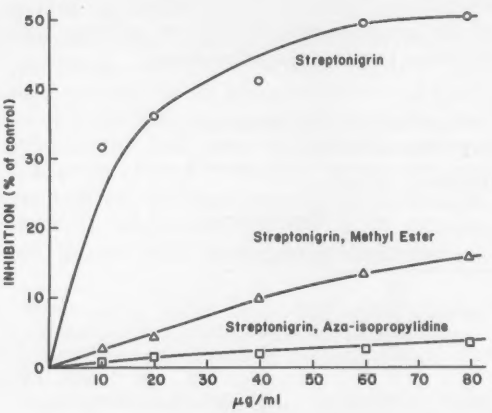

FIGURE 2.—Effect of streptonigrin, streptonigrin methyl ester, and isopropylideneazastreptonigrin on avian myeloblastosis virus DNA polymerase.

However, since recent evidence indicates that DNA polymerase from avian oncogenic viruses may differ biochemically from that from mam-

*Rifamycin SV: NSC-133100; CAS reg. No. 6998-60-3; 2,7-(epoxypentadeca[1,11,13]trienimino)naphtho-[2,1-*b*]furan-1,11(2*H*)-dione, 5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-, 21-acetate.

malian oncogenic viruses, it will be important to compare the viral and DNA polymerase inhibitory activity of streptonigrin within one mammalian oncornavirus system.

The in vivo activity of the drug is extremely interesting in that an ongoing viral oncogenic process has been inhibited. Therefore, the drug must be not only inhibiting recruitment of new target cells but also eliminating the cells already transformed. Experiments in progress indicate that this may indeed occur.

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Preliminary Clinical Trials of Butocin (NSC-172755) in Patients With Generalized Carcinoma of the Breast [1,2]

O. Andrysek,[3] Z. Dienstbier,[3] M. Semonský,[4] and J. Zámečník [5]

SUMMARY

A new Czechoslovakian cytostatic agent, butocin, was tested in in vitro pilot trials, and a relatively high incidence of sensitivity was found in breast, ovarian, and bronchogenic carcinomas. In a group of 14 patients with generalized breast carcinoma, the clinical efficacy of butocin was tested at an average daily dose level of 1 g, given either alone or combined with other cytostatic agents and hormones. No toxic side effects have been observed even after long-term medication. In ten patients an objective regression of the tumor to less than 50% of its original size was observed; in another three patients improvement not amenable to objective appraisal was achieved. One patient did not respond to the treatment, and her state progressively deteriorated.

[Cancer Chemother Rep 57:311–318, 1973]

Butocin belongs to the group of *N*-[δ-(6-purinylthio)valeryl]amino acids and analogous *N*-substituted derivatives of dipeptides and tripeptides (1,2) which might be expected to have antineoplastic application as transportable forms of the carcinostatically active drug, buthiopurine, or of 6-mercaptopurine and 3-(purine-6-ylthio)propionic acid (3) as metabolic cleavage products of buthiopurine.[6] Whereas buthiopurine and 6-mercaptopurine have been found to be therapeutically active in animals with many of the transplantable tumors used, butocin has been active only in H-strain mice with Crocker's sarcoma 180 (S180). An analogous selectivity of action in animals with different tumors was also found in structural combinations of buthiopurine with some other amino acids and peptides (1). The chronic toxic effects of butocin and buthiopurine, in pilot tests done in mice receiving single daily oral doses of 600 mg/kg of each compound, were approximately equal (LD50, 33 and 30 days respectively). In comparative studies of the absorption, distribution, and elimination of orally administered ^{35}S-labeled butocin and ^{35}S-labeled buthiopurine in H-strain mice with S180, it was found that 3 hours after administration the concentration of butocin in the lung tissue was about 100 times as high and the concentration in the tumor tissue was about three times as high as the corresponding concentrations of buthiopurine; both compounds also differed statistically significantly in their elimination rates (4). So far, the results of preliminary clinical trials of butocin in patients with acute hemoblastoses have been published; the drug has exhibited therapeutic

[1] Received Jan 4, 1973; revised May 14, 1973; accepted May 24, 1973.

[2] Butocin: CAS reg. No. 22181-94-8; glycine, *N*-[1-oxo-5-(1*H*-purin-6-ylthio)pentyl]-, ethyl ester; estyl *N*-[δ-(6-purinylthio)valeryl]glycinate.

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[6] Buthiopurine: NSC-130678; CAS reg. No. 608-12-8; valeric acid, 5-(purine-6)-ylthio)-.

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate.

3-(Purine-6-ylthio)propionic acid: NSC-22242; CAS reg. No. 608-10-6; β-(6-purinylthio)propionic acid.

efficacy particularly in patients in the blastic phase of chronic myeloid leukemia (5).

Along with other promising cytostatic agents of both Czechoslovakian and foreign origin, we tested the sensitivity of individual types of human tumors to butocin, using an in vitro method based on the depression of the incorporation of ^{125}I-iododeoxyuridine (^{125}I-5-iodo-2′-deoxyuridine) according to Andrysek et al (6). Butocin was tested in 46 patients with various tumors. Figure 1 shows the percentages of sensitive, minimally sensitive, and resistant patients. There were nine patients with ovarian carcinoma (five sensitive); four with bone tumor (two sensitive); 12 with pulmonary, predominantly bronchogenic carcinoma (seven sensitive); five with digestive tract tumor (two sensitive); and 16 women with breast carcinoma (seven sensitive). The sensitivity of breast carcinoma to cytostatic agents in current usage compared to the sensitivity to butocin is represented in figure 2. These findings encouraged our tests of the clinical efficacy of this cytostatic agent in generalized carcinoma of the breast.

The first results obtained in this pilot study are the subject of the present communication. In addition, basic studies of the efficacy of butocin in ovarian tumors and bronchogenic carcinomas have been or are about to be started.

METHODS

The first appraisal of results was done in 14 women operated on for breast carcinoma. Postoperatively, most were treated with actinotherapy, hormonal therapy, and cytostatic chemotherapy. A majority of these women had been recommended to our care in a generalized stage of disease and in a situation in which classical treatment was unable to stabilize the patient's state or to achieve a remission. In some of the patients (those whose vital tumor cells were available [mostly those with pleural exudate]), the sensitivity to various cytostatic agents was tested, and if the cells were found to be sensitive to butocin, therapy with this medication was started. A fair palliative effect having been established, further patients (those in whom no tumor cell samples were obtainable in a simple way) were treated with butocin without previous selection by an in vitro sensitivity test. Butocin given alone or concurrently with symptomatic drugs was administered to seven patients; the remaining seven patients received butocin combined with other cytostatic treatments. In the first two patients of the latter group of seven, the combination therapy was motivated ethically because the clinical efficacy of butocin had not been established at that time. In the remaining five patients, the physical state had deteriorated during previous systemic cytostatic treatment; improvement was observed only when butocin had been added. We are now convinced that in all similar instances the previous, inefficient treatment should be discontinued and butocin monotherapy should be instituted instead.

The dosage followed a standard pattern: during the first week, each patient received 1 tablet (0.5 g) three times daily. Afterwards, 1 tablet was given twice daily (ie, 1 g of butocin daily) continuously until a relapse occurred indicating a loss of clinical sensitivity of the tumor to the preparation. At the time of the present evaluation, some of the patients have been maintained on butocin medication.

Because the preparation is supplied in tablet form, we chose a simple dosage pattern not requiring conversion of dosage in relation to square meter of body surface area. The dosage used corresponds approximately to 1 g/m^2/day.

Whenever possible, we used objective criteria in appraising the therapeutic effect, ie, reduction of tumor masses, especially reduction of the formation of pleural exudate; decrease in the size of metastases visible on lung roentgenograms or liver scintiscans; and changes in skeletal roentgenograms or in the ^{85}Sr uptake in the areas of bone metastases. If possible, we verified the effect surgically. Subjective improvement was indicated if the patient was able to discontinue the use of opiates for pain, was restored to normal walking capacity, experienced a normalization of the life conditions (in bedridden patients with multiple bone metastases), or had an improvement in the general clinical state.

Toxic side effects of butocin were appraised with regard to clinical findings, changes in the blood cell counts, and results of function tests of the liver, digestive tract, and urinary tract.

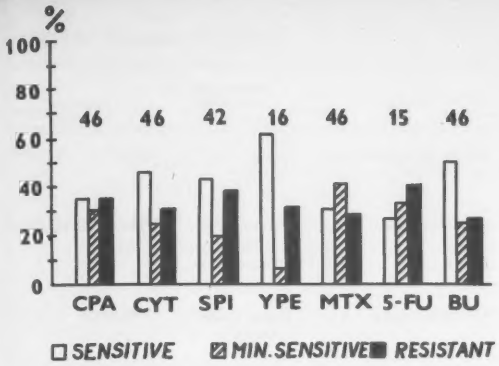

FIGURE 1.—Results of in vitro tests for sensitivity to various cytostatic agents in 46 patients with miscellaneous cancers including breast carcinoma. Columns indicate proportions of patients who were sensitive, nonsensitive, and resistant to the given agent, expressed in terms of percent of the number of examinations given above each column. CFA = cyclophosphamide; CYT = sodium bromebrate; SPI = SPI-77; YPE = 5-([bis(2-chloroethyl) amino]methyl) uracil, hydrochloride; MTX = methotrexate; 5-FLU = 5-fluorouracil; BU = butocin.

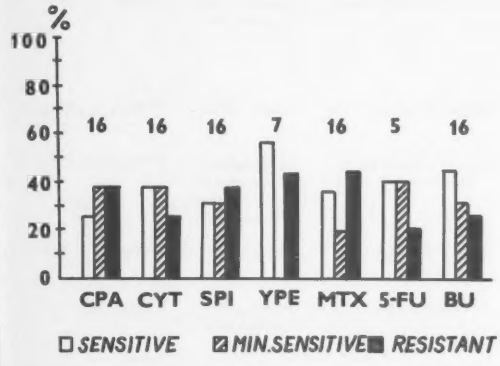

FIGURE 2.—Results of the in vitro sensitivity to various cytostatic agents of patients with breast carcinoma. *See* fig 1 legend for drug names.

RESULTS

Table 1 presents a survey of the results obtained in the first 14 patients treated with butocin alone or in combination. These patients, all of whom were in generalized stages of the disease, were treated from 1970 to 1972. Their ages averaged 59 years (range, 36–81 years; median, 38.5 years). Breast carcinoma had been diagnosed before climacterium in four patients and during climacterium in one patient. In the remaining patients, the disease was diagnosed during the menopause. The time elapsed between the occurrence of the disease (and surgical treatment) and the admission to our care averaged 3 years and 9 months (range, 1.5–6.5 years; median, 4 years). Multiple metastases were objectively proved to be present only in bones in one patient and only in soft tissues in five patients; in the remaining eight women, multiple metastases were proved to be present in both bones and soft tissues. We resorted to butocin medication only when the previous treatment was no longer effective. The total dose per patient averaged 181 g of butocin (range, 58–704 g). The total dose necessary for producing an objective effect averaged 45 g (range, 10–190 g). The period of treatment was from the start of the medication until an effect was noted (average, 41 days; range, 7–180 days; median, 103 days). We have recorded no objective toxic side effects of butocin treatment nor have the patients experienced subjective complaints of such effects, with the exception of leukopenia in one patient (No. 5) who had been admitted for treatment with leukopenia which had occurred after previous treatment (3000 cells/mm³). Although a certain effect was produced, a week before death her leukocyte count dropped to 1200 cells/mm³.

The preparation was therapeutically effective for an average of 133 days (range, 40–396 days; median, 218 days). Seven patients are being maintained on the medication at the time of this report and have so far remained in remission. The duration of remission in the patients whose treatment with butocin had been discontinued (due to a loss of sensitivity and transfer to a different medication or to death) averaged 188 days (median, 218 days).

Among the 14 patients treated, an objective effect manifested by a reduction of tumor masses by more than 50% of the original size (always accompanied by an excellent subjective effect) was proved in ten patients; three patients experienced a fair subjective effect, without the possibility of an objective appraisal of the drug effect, or a partial regression of the tumor (indicated as $<50\%$ in table 1). In only one patient was no drug effect seen, and during

TABLE 1.—Results of butocin

Patient No.	Age	Date of diagnosis	Diagnosis	Date and type of surgery	Histologic type	Metastatic spread at admission	Previous treatment*
1	36	May 1968	Breast carcinoma, Paget's disease (left side)	May 1968, radical mastectomy + axilla	Carcinoma (Paget's cell)	Lungs	^{60}Co
2	42	Dec 1966	Breast carcinoma (right side)	Apr 1967, radical mastectomy + axilla	Adenocarcinoma	Axillae, mediastinum, bones, lungs, liver, exudate	TST, CYC, castration
3	59	June 1970	Breast carcinoma (left side)	June 1970, radical mastectomy + axilla	Adenocarcinoma	Bones, lungs, pleura	^{60}Co, TST
4	59	July 1966	Breast carcinoma (right side)	Aug 1966, radical mastectomy + axilla	Scirrhous, cystadenocarcinoma	Bones, liver, skin, lungs (cachexia)	^{60}Co, CYC, TST
5	59	1967	Breast carcinoma (left side)	1967, enucleation of tumor	Solid carcinoma (necropsy)	Mediastinum, liver, bones, pleura (cachexia)	^{60}Co, CYC (9 g), TST
6	65	Nov 1965	Breast carcinoma (left side)	Nov 1965, radical mastectomy + axilla	Adenocarcinoma	Bones, mediastinum, pleura	^{60}Co, CYC, TST, hypophysectomy
7	48	Aug 1969	Breast carcinoma (right side)	Sept 1969, radical mastectomy	Diffuse infiltrative carcinoma	Mesogastrium, liver (ileus)	^{60}Co, TST, CYC
8	81	Sept 1966	Breast carcinoma (left side)	Sept 1966, radical mastectomy (left side); June 1968, radical mastectomy (right side)	Cribriform carcinoma	Lungs, lymph nodes, skin	DES
9	60	May 1970	Breast carcinoma (right side)	May 1970, radical mastectomy + axilla	Adenocarcinoma	Bones, lungs	X-ray irradiation, TST, CYC
10	73	Feb 1970	Breast carcinoma (left side)	Feb 1970, radical mastectomy	Diffuse infiltrative carcinoma	Right breast, skin, thorax, pleura, bones, lungs (cachexia)	^{60}Co, TST, CYC
11	59	Nov 1967	Breast carcinoma (right side)	Jan 1968, radical mastectomy (left side)	Diffuse infiltrative carcinoma	Bones	^{60}Co
12	77	June 1970	Breast carcinoma (left side)	June 1970, radical mastectomy (left side)	Adenocarcinoma	Bones, liver, lungs, skin (cachexia)	TST, CYC
13	54	Mar 1969	Breast carcinoma (left side)	Mar 1969, radical mastectomy (left side) + axilla	Adenocarcinoma	Pleura	^{60}Co
14	62	Apr 1970	Breast carcinoma (left side)	Apr 1970, radical mastectomy + axilla	Scirrhous carcinoma	Liver, bones, ascites (cachexia)	^{60}Co

*TST = testosterone; CYC = cyclophosphamide; DES = diethylstilbestrol; VBL = vinblastine; PAR = paroxypropione.
†On the day of assessment both treatment and drug effect were continuing.
‡ST = symptomatic treatment.

the treatment her disease slowly progressed. An appraisal of those seven patients who had received butocin alone without the addition of hormonal or other cytostatic drugs shows that in four cases the tumors were objectively reduced to less than 50% of their original size; the remaining three patients likewise experienced both subjective and objective improve-

Butocin therapy							
From	To	Total dose (g)	Side effects	Concurrent treatment‡	Effect of treatment	Duration of effect (days)	Sensitivity to ^{125}I-iododeoxyuridine
6/24/70	3/29/72	704		PAR (240 tablets), CYC (16.2 g), castration	Objective (> 50%) within 6 months; subjective+ within 3 weeks	396	Not done
11/22/71	9/10/72	296		CYC (15.2 g), castration	Objective (> 50%) within 28 days; subjective+ within 21 days	265	Sensitivity+
12/ 3/71	6/30/72	225		^{198}Au intrapleurally	Objective (> 50%) within 7 days; subjective+ within 7 days	200	Sensitivity+
1/14/72	4/ 9/72	87		ST	Objective (> 50%) and subjective+ within 14 days	76	Not done
1/26/72	4/14/72	78	Terminal leukopenia	ST	Objective (< 50%) and subjective+ within 30 days	40	Not done
3/ 1/72	9/20/72	190		VBL (20 mg), CYC (3 g)	Objective (> 50%) and subjective+ within 42 days	156	Sensitivity+
3/ 6/72	9/20/72	200		ST	Objective (> 50%) and subjective+ within 14 days	184	Not done
4/ 3/72	9/20/72	170†		ST	Objective (< 50%) and subjective+, at present stationary after 60 days	112†	Not done
5/17/72	9/20/72	100		X-ray irradiation	No effect	—	Not done
5/24/72	9/20/72	116†		ST	Objective (> 50%) within 60 days; subjective+ within 21 days	58†	Not done
6/12/72	9/20/72	107†		CYC (6 g), VBL (60 mg), TST (6 g)	Objective (> 50%) and subjective+ within 30 days	70†	Not done
6/15/72	9/20/72	97†		ST	Objective (< 50%) and subjective+ within 14 days	84†	Not done
6/30/72	9/20/72	75†		^{198}Au intrapleurally	Objective (> 50%) and subjective+ within 14 days	60†	Sensitivity+
7/23/72	9/20/72	58†		ST	Objective (> 50%) and subjective+ within 21 days	58†	Not done

ment, but it was impossible to objectively assess the extent of the reduction of the tumors.

CASE REPORTS

Patient No. 4.—Patient No. 4, born in 1913, was diagnosed with scirrhous carcinoma of the right breast, and in August 1966 she underwent a radical mastectomy with exenteration of the axilla. After surgery, she received repetitive

radiation therapy with radioactive cobalt and chemotherapy with cyclophosphamide and paroxypropione.[7] Butocin administration was started on January 14, 1972. Objective findings included metastases in bones; pathologic fracture of the right clavicle; roentgenographically proved extensive affliction of the pelvis, lumbar spinal column, and both femurs; further metastases in the liver and lungs; multiple skin metastases on the right half of the thorax; and conspicuous lymphatic edema of the right arm. The patient was cachectic and immobile, and she received opiates as palliatives. At that time, mild bone marrow depression after previous treatment with cyclophosphamide was present (4000 leukocytes/mm^3). Butocin monotherapy was started at a dose level of 1 tablet three times daily; after 14 days the patient was no longer receiving analgesics, she began to walk, and she complained only of an increasing edema of the right upper limb. Within the next 14 days her mobility further markedly improved, pains at the bone metastatic sites subsided, both physical and roentgenographic findings in the lungs improved, and the multiple skin metastases showed a slight reduction in size. After 6 weeks, the improved mobility was accomplished by a subsidence of the right arm edema, a marked regression of skin metastases, and granulation in an extensive disintegrated focus at the site of the postoperative cicatrization. The patient remained in this considerably improved state, reporting only moderate pains in the osseous foci at a change in the weather (reported also by the other patients), until April 7, 1972, when she experienced sudden dyspnea. Roentgenograms exhibited signs of intense lymphatic infiltration of both lung fields, and the patient died 2 days later.

Patient No. 7.—Patient No. 7, born in 1924, became ill in August 1969; at surgery (radical right mastectomy) histologic findings showed diffuse infiltrative carcinoma. Postoperative treatment consisted of cobalt irradiation, cyclophosphamide, and paroxypropione. In September 1971, vomiting and nausea leading to ileus occurred; surgery revealed an extensive tumor in the left mesogastrium about 15 cm in diameter, originating from the retroperitoneal space, fixed to the lumbar spinal column, and growing through the transverse colon. The abdominal cavity was filled with exudate containing clusters of tumor cells whose identified structures corresponded to breast adenocarcinoma. Palliative transversostomy was performed. After 14 days the patient's general state had improved to the extent that it permitted transverso-sigmoideoanastomosis to be performed. The tumor, inoperable because of its extent and growth through the tissues, was left in situ. The patient was dismissed to home care in a poor state with anorexia and a moderate progression in the growth of the palpable tumor. In March 1972, she was admitted to our ward; the palpable 15–17-cm tumor was still present in the left mesogastrium and liver scintiscan indicated suspected metastases. Butocin monotherapy was started; after 14 days the palpable tumor in the mesogastrium was distinctly diminished, and the patient's appetite was improved and she had gained body weight. Butocin medication was continued and after 6 weeks the tumor was no longer palpable. On May 19, 1972, the abdominal cavity was surgically incised in an attempt to radically remove the rest of the tumor. Completely negative findings were obtained for the abdominal cavity and liver, with the exception of a mild thickening of the pancreatic tail, but without any visible signs of pancreatic peritonitis in its vicinity. Likewise, histologic findings were completely negative in all suspect samples taken. This patient had been receiving butocin (1 tablet twice daily) without any toxic signs of intolerance; she gained body weight, felt excellent, and had no signs of any further progression of the disease for some time. In December 1972, palpable tumors appeared in the abdominal cavity. Further laparotomy revealed a generalized metastatic process in the abdominal cavity and liver, histologically identified as anaplastic carcinoma. After treatment with cyclophosphamide, sodium

[7] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

Paroxypropione: NSC-2834; CAS reg. No. 70-70-2; propiophenone, 4'-hydroxy-.

bromebrate, and SPI-77,[8] an almost complete regression of the metastatic spread was achieved, and the patient, in a good state, was retransferred to maintenance treatment with 1 g/day of butocin.

Patient No. 10.—Patient No. 10, born in 1899, underwent ablation of the left breast for diffuse infiltrative breast carcinoma in February 1970. In April 1970, she received postoperative irradiation. Upon admission to our study, she had local relapse of the tumor, tumorous infiltration of the right breast, infiltration of the left and partial infiltration of the right hemithorax skin, and multiple metastases in the spinal column, pelvis, and both femurs. Ambilateral pleural exudate, cachexia, and leukopenia (2700 cells/mm^3 after previous treatment) were also noted. On May 24, 1972, butocin therapy was started (1 tablet twice daily). After 2 months, axillary metastases diminished, fixation of the right breast loosened, exudate disappeared from both pleural cavities, and bone pain subsided; at this time the patient is capable of walking unaided, her leukogram has improved, she is feeling excellent, and she reports continuous moderate subjective improvement in her physical state. She continues to receive butocin therapy.

DISCUSSION

We have attempted to summarize the short-term therapeutic results obtained in our first group of 14 patients with generalized breast carcinoma. Despite the small number of patients followed so far, we are surprised at the high percentage of objective positive effects and the dramatic course and fast onset of the effects achieved with butocin even at stages where any therapy whatever is usually abandoned and the patients receive only symptomatic treatment. This is why we decided to publish our observations in the present preliminary communication, regardless of the brevity of the followup periods.

In view of these facts, the conclusions to be presented also have only preliminary validity.

Butocin, given at a daily dosage level of 1–1.5 g, is practically nontoxic. One of our female patients received a total dose of 704 g without any toxic manifestations. In several instances, we started butocin medication in patients with bone marrow depression induced by other cytostatic agents, and we observed a rapid improvement in the blood cell count. We ascribe the preterminal bone marrow depression noted in one of our patients (No. 5) to her previous intense long-term treatment with alkylating agents and repetitive irradiations, not to butocin.

During butocin treatment, we observed a relatively early onset of improvement in the patients' subjective state, associated with improved mobility and a speedy restoration of the walking ability. In most instances these changes were also objectively proved by findings of tumor regression.

The daily dose of 1.5 g (approximately corresponding to 1 g/m^2) during the first week and 1 g during subsequent maintenance therapy was chosen on the basis of conclusions made in the search for an optimal dosage pattern (first administration of the drug to man not included in the present communication). In responsive patients, we often saw a rapid regression of metastases at these dosage levels (in one instance this was proved by necropsy after a 3-day administration). However, because we fear potential complications at higher dosage levels, we reduced the dosage by one third after the first week. On the other hand, in resistant patients even elevated doses of butocin produced no effect. The simple dosage pattern was also chosen because of the tablet form of butocin and with a view to its usage in outpatients.

During the rapid reduction in size of metastases, we registered no undesirable side effects (intoxication, elevated uricemia, fever, colliquation, etc). The remissions produced by butocin vary in duration; in very advanced cases the action of this preparation ends by a sudden turn to the worse and death (*see* case report for patient No. 4). As yet, we are not in a position to offer any definite opinion concerning the duration of remissions, because the

[8] Sodium bromebrate: NSC-104801; CAS reg. No. 25550-61-2; acrylic acid, 3-*p*-anisoly-3-bromo-, sodium salt.

SPI-77: NSC-72274; CAS reg. No. 1508-45-8; podophyllic acid, 2-ethylhydrazide.

followup periods have been short, the therapy is currently continuing, and the number of patients has been low. The fact that in our group of patients we found a percentage of clinical efficacy far exceeding that found in the patients tested in vitro (6,7) is accountable to the error inherent in small numbers. All female patients who had responded positively when in vitro tests of butocin had been given before the start of the medication also responded positively to the treatment. Nevertheless, we suppose that an extended study will reveal more cases of clinically unsensitive patients, in better correspondence with the in vitro findings than the high percentage of clinical efficacy presently reported. We do believe, however, that the effect of butocin on generalized breast carcinoma resistant to any previous hormonal, radiation, or cytostatic therapy described by us is so remarkable that it justifies a clinical controlled therapeutic trial of butocin as well as a more profound study and analysis of its therapeutic efficacy in a larger number of patients, even with less-advanced breast tumors.

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ADDENDUM

At the time of the last revision of this article (Aug 1973), preliminary results of the therapy with butocin could be evaluated in an additional 20 patients with generalized breast cancer. Most of them were treated with butocin only, under the same conditions as described in this text. An objective regression of the tumor size by more than 50% was achieved in 12 patients, three showed a partial remission of the disease, and the remainder had no effect. The positive therapeutic effect has persisted an average of 1 year.

Treatment of Acute Leukemia With 5-Azacytidine (NSC-102816) [1,2,3]

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SUMMARY

5-Azacytidine was administered to 28 patients with acute leukemia and four patients with chronic myelogenous leukemia in blast cell crisis, all but one of whom had received extensive prior therapy. Four complete and four partial remissions were achieved in 29 patients evaluable for response. The median duration of complete remission was 19 weeks. Nausea, vomiting, and diarrhea were common. Hypotension following administration of the drug occurred in four patients, and in one case this was fatal.

[Cancer Chemother Rep 57:319–323, 1973]

5-Azacytidine is a pyrimidine nucleotide analog produced microbiologically by a species of *Streptoverticillium* which was initially isolated in Czechoslovakia (1). It closely resembles cytidine in chemical structure and is incorporated into ribonucleic acids, affecting both DNA and RNA synthesis (2). In vitro, it inhibits RNA synthesis by the blocking of orotodylic acid decarboxylase (3). The drug is converted to a nucleotide by uridine kinase and is subsequently phosphorylated and incorporated into RNA; this modified structure inhibits protein synthesis (4). The diphosphate is a substrate of the nucleotide reductase and, subsequently, DNA pronase; thus, the drug is also incorporated into DNA. In cell culture systems, there is evidence suggesting that the drug has some cell-cycle-nonspecific activity as well as cell-cycle-specific activity (5). In animal systems, the mechanism of resistance is a reduction in the activity of uridine kinase and a subsequent reduction in the conversion of 5-azacytidine to 5-azacytidine-5′-phosphate.

5-Azacytidine has been reported to be active in tumors resistant to cytosine arabinoside, vincristine, 6-mercaptopurine, 5-hydroxypicolinaldehyde thiosemicarbazone, and BCNU. In experimental animals, it is synergistic with both L-asparaginase and 6-thioguanine (5).

Previous studies have shown that this drug is active in a number of solid tumors and in acute leukemia. The original reports from Czechoslovakia showed encouraging results in the treatment of acute leukemia; however, these patients received other drugs known to be active against leukemia, and assessment of the activity of 5-azacytidine alone is difficult (6). Activity against solid tumors has been shown in phase I and II studies with objective responses

[1] Received Mar 8, 1973; revised May 11, 1973; accepted May 24, 1973.

[2] Supported in part by grants CA–05831, CA–11520, and CA–10376 from the National Cancer Institute (NCI), National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

[3] 5-Azacytidine was supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[4] Department of Developmental Therapeutics (Drs. McCredie, Bodey, Burgess, Gutterman, Rodriguez, and Freireich) and Department of Pediatrics (Dr. Sullivan), The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston.

occurring in patients with cancer of the breast, in patients with melanoma, and in patients with cancer of the colon (7).

This study was designed to test the efficacy of 5-azacytidine when used alone in the treatment of patients with acute leukemia (regardless of age or cell morphology) who had active disease following chemotherapy with other antileukemic agents.

MATERIALS AND METHODS

A total of 32 patients were entered into the study; 31 of these patients had had extensive prior therapy, given either in combination or sequentially. One patient with chronic myelogenous leukemia in blast crisis had had no previous chemotherapy. The patients ranged in age from 1 to 72 years (median age, 27 years), and 20 of the 32 patients were male. Eighteen of the patients had acute myeloblastic leukemia, four had chronic myelogenous leukemia in blast crisis, and ten had acute lymphoblastic or undifferentiated leukemia.

The initial starting dose of 5-azacytidine was 400 mg/m²/day × 5; it was selected after a phase I study in patients with solid tumors had shown this dose to be well tolerated.[5] The drug was supplied as a white powder in 50-mg vials and was reconstituted in water and mixed with 50–200 ml of 5% dextrose in water immediately prior to use. It was administered intravenously (iv) for 15–30 minutes daily for 5 days. Twenty-seven of the 32 patients received an initial starting dose of 400 mg/m², three received a starting dose of 300 mg/m², and two received a starting dose of 150 mg/m². The second course was administered on Day 15, and doses were modified as required. The dose was escalated in the second course for five patients and reduced for four patients. One patient received 400 mg/m² for the first course, 500 mg for the second course, and 600 mg for the third course prior to achieving complete remission. For nine patients, the initiation of the third course was delayed for 3 weeks because of bone marrow hypocellularity (less than 5%). Two patients required the reinstitution of 5-azacytidine therapy earlier (one on Day 11 and one on Day 14) because of increasing disease in the bone marrow and peripheral blood.

Complete remission is defined as less than 10% blasts and promyelocytes in the bone marrow and less than 5% blasts, a hemoglobin value greater than 11 g/100 ml, a granulocyte count greater than 1000 cells/mm³, and a platelet count greater than 100,000 platelets/mm³.

RESULTS

Of the 32 patients who were entered into this study, 29 were evaluable. Three patients were not considered to be evaluable for response because they were withdrawn from the study immediately after the first course of chemotherapy (at the parents' request). Eight patients had a hematologic response, four achieving complete remission. Seven patients died within the first 3 weeks after the start of chemotherapy. Eight patients had less than three courses of chemotherapy and were considered to have had inadequate trials. Six patients failed to respond.

The response rate in patients who were evaluable for response was 28% (eight of 29 patients), with complete remission being achieved in 14% (four of 29 patients) (table 1). The response rate for those patients receiving adequate trials of chemotherapy was 57%, and the complete remission rate for those receiving adequate trials was 28%.

When the response is broken down by diagnosis (table 2), it can be seen that most of the responses occurred in patients with acute myeloblastic leukemia. Only one patient with acute lymphoblastic or acute undifferentiated leukemia achieved a complete remission. None of the patients with acute lymphoblastic leu-

TABLE 1.—Response to treatment with 5-azacytidine

No. of responses/No. of evaluable patients	8/29	(29%)
No. of complete responses/No. of evaluable patients	4/29	(14%)
No. of responses/No. of adequate trials	8/14	(57%)
No. of complete responses/No. of adequate trials	4/14	(28%)

[5] McCredie KB, and Gottlieb JA. Unpublished observation.

TABLE 2.—Diagnosis and response to treatment with 5-azacytidine

Diagnosis	Complete response	Partial response	Total No. of responses/ No. of patients with diagnosis (%)
Acute myeloblastic leukemia	3	4	7/18 (39)
Acute lymphoblastic leukemia	1	0	1/10 (10)
Chronic myelogenous leukemia-blast crisis	0	0	0/4 (0)

kemia had a partial response, and none of the four patients with chronic myelogenous leukemia in blast crisis responded.

The median duration of therapy for the evaluable patients was 42 days, with a range of 10–294 days. The median duration of therapy for those patients who failed to respond to chemotherapy was 30 days, with a range of 10–84 days. The median duration of response for those patients who responded to chemotherapy was 173 days, with a range of 59–294 days. In the four patients who achieved complete remission, the durations of chemotherapy were 166, 181, 247, and 294 days. The median duration of complete remission was 136 days (range, 73–200 days).

Maintenance therapy with intermittent doses of 5-azacytidine was difficult because of profound and often prolonged myelosuppression. Three of the four patients who achieved complete remission had delays of 3 months or more in the introduction of maintenance therapy because of severe granulocytopenia and thrombocytopenia. The myelosuppression in these patients was complicated by infection which required hospitalization and intensive therapy with antibiotics and leukocyte transfusions. The delays in the start of remission maintenance therapy were 90, 102, and 114 days. Maintenance therapy was started after recovery from this myelosuppression at the time or shortly after complete remission was observed.

In two patients, platelet transfusions were required for 25 of the 102 days and 32 of the 114 days. Both of these patients had granulocytopenia with total peripheral granulocyte counts of less than 1000/mm^3 for 40 and 42 days, respectively. Both patients experienced episodes of septicemia, one with *Klebsiella* sp. and *Candida albicans*, and the other with *Aeromonas* sp. These conditions were eventually controlled with appropriate antibiotic therapy and supportive care with granulocytes prior to the recovery of normal peripheral granulocyte counts.

The third patient had a delay of 3 months before the next course of chemotherapy, and required hospitalization because she had a fever of unknown origin. Positive blood cultures were not obtained; however, she had infected thrombosed hemorrhoids, and a pure growth of *Proteus mirabilis* was isolated. She responded satisfactorily to appropriate antibiotic therapy and white blood cell transfusions.

In the fourth patient, there was a 2-month delay between the second and third courses of chemotherapy, and hospitalization was required for 3 weeks because of thrombocytopenia and granulocytopenia. Platelet transfusions were started on Day 12, and prophylactic antibiotic therapy was started; no positive cultures were obtained despite persistent fever. After the patient recovered, the fourth course of chemotherapy was started in 45 days without marked myelosuppression, and the fifth course was started 30 days later.

All patients treated with 5-azacytidine experienced nausea, and most patients experienced vomiting and diarrhea (table 3). These symptoms were severe enough to cause marked anorexia in the majority of patients, and a few patients required iv therapy to prevent dehydration. Sixty percent of the patients receiving a rapid iv infusion of 5-azacytidine experienced fever with elevations in body tem-

TABLE 3.—Toxic effects of 5-azacytidine

Toxic effect	Rate of occurrence (%)
Nausea	100
Vomiting	90
Diarrhea	65
Fever	60
Bone marrow hypoplasia	25
Hypotension	12

perature to 104 F. The fever usually occurred 1–4 hours after the administration of the drug and continued each day for the duration of the course. However, body temperatures fell to normal levels within 24 hours of the completion of the course. On the whole, the drug was better tolerated with the second course of chemotherapy; however, nausea, vomiting, diarrhea, and fever were still major side effects. Severe bone marrow hypoplasia resulting in profound peripheral pancytopenia and the resultant complications of infection and hemorrhage occurred in one quarter of the patients and prevented the administration of the second course in most patients. Hypotension associated with the administration of the drug was seen in four patients; in one patient, it was associated with oliguric renal failure and death.

In the patient that died there was a direct relationship between the infusion of 5-azacytidine and the development of hypotension, tachypnea, and cyanosis. Although treatment was discontinued, the blood urea nitrogen continued to increase, and severe diarrhea associated with the administration of 5-azacytidine caused considerable electrolyte imbalance. Despite vigorous supportive care, this patient died 5 days later. Autopsy showed evidence of disseminated *Candida tropicalis* infection in the kidneys, adrenal glands, gastrointestinal tract, and lungs; this infection was unsuspected prior to the patient's death.

DISCUSSION

In this study, 5-azacytidine was used to treat 32 patients with acute leukemia. The patients who were selected for this study were in all age groups and, with the exception of one patient with chronic myelogenous leukemia in blastic crisis, had advanced disease which either had failed to respond or had relapsed with conventional forms of combination chemotherapy. The drug has been shown to be considerably active in patients with acute myeloblastic leukemia, as well as in patients with acute lymphoblastic disease. Although activity was not seen in patients with chronic myelogenous leukemia in blast crisis, all but one of these patients had had extensive prior therapy, and the course in all cases was complicated by marked myelosuppression and death.

The toxic effects experienced by this group of patients were marked (particularly the myelosuppression), and maintenance of remission with this drug alone is complicated. The toxic effects have been lessened by a reduction in starting dose from 400 to 300 mg/m²/day × 5, each daily dose being divided into three daily infusions given over 30 minutes. When given by this regimen, the drug appears to be better tolerated, and the hypotension, nausea, vomiting, and diarrhea appear to be lessened.

With the synergism shown in laboratory animals, 5-azacytidine may well have a role in future combination chemotherapeutic regimens for the treatment of acute leukemia of both adults and children. These combinations may be better tolerated for remission induction and may also allow for more effective remission maintenance therapy.

LIST OF COMPOUNDS

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3

5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

5-Hydroxypicolinaldehyde thiosemicarbazone: NSC-107392; CAS reg. No. 19494-89-4

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Phase II Clinical Trials of BCNU (NSC-409962) and Bleomycin (NSC-125066) in the Treatment of Kaposi's Sarcoma [1,2,3]

Charles L. Vogel, Dennis Clements, Alex K. Wanume, Thomas Toya, Aron Primack, and Sebastian Kyalwazi [4]

SUMMARY

Phase II clinical trials of BCNU and bleomycin were done in patients with Kaposi's sarcoma. Objective antitumor responses were seen in nine of 21 patients (43%) treated with BCNU and six of ten (60%) treated with bleomycin. Responses to BCNU were more complete and durable than the responses to bleomycin. BCNU toxic effects at dose levels of 200 mg/m² (repeated every 6–8 weeks) were tolerable; however, there was one drug-related death in a patient who received higher dose levels. Bleomycin given at a total dose of 300 mg over a 1-month period was well tolerated; however, one patient treated with 600 mg died of pulmonary fibrosis. Several agents, including BCNU, bleomycin, NSC-45388, vincristine, and actinomycin D, have now been shown to be effective against Kaposi's sarcoma. It is hoped that various combinations of these agents can be used to obtain more complete control of this disease in Uganda where radiation therapy is not yet available.

[Cancer Chemother Rep 57:325–333, 1973]

Kaposi's sarcoma is extremely sensitive to both X-rays and chemotherapy. In previous studies done in Uganda it was shown that actinomycin D either alone (1,2) or in combination with vincristine (3) is effective in inducing remissions in Kaposi's sarcoma. Subsequently, it was shown that NSC-45388 could produce responses in 50% of the patients whose diseases were refractory to treatment with actinomycin D (4). The present study reports on the effectiveness of BCNU and bleomycin in various clinical and pathologic types of Kaposi's sarcoma.

PATIENTS AND METHODS

Twenty-one men hospitalized at the Solid Tumor Center (Kampala, Uganda) with histologically proven Kaposi's sarcoma were entered into the BCNU study. Of these patients, four were primarily treated with BCNU for the indolent, nodular form of the disease while the remaining patients received BCNU after treatment with either actinomycin D or after an actinomycin D-plus-vincristine combination did not control the disease. Ten men with refractory tumors were treated with bleomycin; five of these patients had received BCNU previously.

[1] Received Mar 20, 1973; revised May 23, 1973.

[2] Supported by Public Health Service contract PH43–67–1343 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] BCNU and bleomycin were supplied by Cancer Therapy Evaluation, DCT, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[4] Solid Tumor Center of the Uganda Cancer Institute and Department of Surgery, Makerere University Medical School, Kampala, Uganda.

Pretreatment evaluation included measurements and clinical photography of evaluable lesions, a complete blood count (cbc), and roentgenograms of the chest and extremities. Clinical and histologic characteristics of the lesions are described in accordance with the classification scheme of Taylor et al (5), a summary of which appeared in a previous issue of this journal (4).

BCNU was kept frozen at –20 C, and immediately prior to being given, it was dissolved in 3 ml of ethanol, diluted to 50 ml with sterile isotonic saline, and administered by rapid intravenous (iv) infusion within 10 minutes of preparation. Five patients (Nos. 67, 88, 93, 173, and 183) received a first course of BCNU at a dose level of 125 mg/m² for 3 days while all subsequent courses for these and all other patients were given in a single dose of 200 mg/m². BCNU courses were repeated every 6–8 weeks depending on recovery from hematologic toxic effects. All responding patients were treated, when possible, with five or six full courses of treatment. Objective evidence of tumor progression during treatment was considered an indication for termination of the trial and institution of alternative forms of therapy. Bleomycin was dissolved in 10 ml of sterile water for injection and was given at a dose level of 30 mg every 4 days by rapid iv infusion up to a total dose of 300 mg. Other schedules of drug administration were not found to offer any advantage (or disadvantage) to this twice-a-week schedule.[5] A second course was given to only one patient (No. 168) who died, subsequently, of pulmonary fibrosis.

Measurement of evaluable lesions, a cbc, and roentgenograms of bones previously found to be involved with tumors were done just prior to each subsequent course of therapy. Interim blood cell counts between courses of the BCNU therapy were seldom done since most patients lived in remote areas of the country, thus making frequent followup visits expensive and impractical.

A complete response (CR) to treatment was defined as disappearance of all evidence of disease, and a partial response (PR) was defined as greater than a 50% reduction in the size of measurable tumor masses. Occasionally, in patients who had lesions that were difficult to measure, dramatic changes in symptoms or edema were considered as objective partial responses.

[5] Rosenbaum C, and Carter SK. Clinical brochure: bleomycin (NSC-125066, "Bleo," BLM). Prepared by the NCI, Bethesda, Md, Mar 1970.

RESPONSE

BCNU (table 1)

Nine of 21 patients (43%) treated with BCNU had objective signs of tumor regression. Of these, four patients have enjoyed unmaintained complete responses for periods of 1–2 years. Three of these patients were primarily treated for newly diagnosed nodular disease while the fourth (No. 124) had severe aggressive nodular disease with anaplastic histopathology that had proved refractory to actinomycin D and the combination of mechlorethamine, vincristine, prednisone, and procarbazine (MOPP) (6). Of the five patients who had partial responses, two (No. 167 and No. 185) have been well controlled with maintenance BCNU therapy, two relapsed rapidly and were switched to other drugs, and one was lost to subsequent followup. Among the nonresponding patients or those relapsing after an initial response to BCNU, two (No. 67 and No. 254) subsequently responded to NSC-45388 (4) and two (No. 151 and No. 390) responded to bleomycin. There was no consistent correlation between tumor histopathology and response to BCNU. Thus, four of nine patients with mixed cellular tumors, three of eight with monocellular tumors, and one of three with anaplastic tumors responded.

On initial examination of the data in table 1, there appears to be a dose-response relationship, with patients receiving more courses and higher cumulative doses of BCNU responding better than those receiving fewer courses and lower cumulative doses. It should be remembered, however, that most patients who received fewer than three courses of BCNU had had therapy terminated because of objective evidence of tumor progression, while those who received more than three courses had relatively stable lesions or objective signs of tumor regression after two courses.

TABLE 1.—Patient characteristics and responses to BCNU

TABLE 1.—Patient characteristics and responses to BCNU

Patient No.	Age	Previous treatment	Dose level (mg)	Response*	Predominant clinical tumor type	Histologic type	Total courses of BCNU	Total dose (mg)	Response	No. of days to maximum regression	Duration of remission (mos)
67	42	Cyclophosphamide	9750	NR	Infiltrative	Monocellular	3	1224	PR	120	2
		Actinomycin D + vincristine	14.5/6.9	PR							
74	50	Cyclophosphamide	6000	NR	Nodular	Monocellular	3	920	NR	—	—
		Actinomycin D	16.6	PR							
88	70	Actinomycin D	10.5	NR	Nodular	Mixed	2	800	NR	—	—
93	28	Cyclophosphamide	5900	NR	Florid	Mixed	3	1492	NR	—	—
		Actinomycin D	11.5	PR							
120	57	Actinomycin D + vincristine	10/16	CR (2 mos)	Florid	Monocellular	4	1400	PR	131	2
124	22	Actinomycin D	15	PR	Aggressive	Anaplastic	6	1680	CR	197	12+ (unmaintained)
		MOPP†	2 courses	NR	Nodular						
151	45	Actinomycin D + vincristine	15/6	CR (7 mos)	Florid	Anaplastic	1	350	NR	—	—
		Actinomycin D + vincristine	10/10.5	NR							
167	50	Actinomycin D	20	PR	Infiltrative	Mixed	10	3000	PR	150	14+ (maintained)

TABLE 1.—Patient characteristics and responses to BCNU—Continued

Patient No.	Age	Previous treatment	Dose level (mg)	Response*	Predominant clinical tumor type	Histologic type	Total courses of BCNU	Total dose (mg)	Response	No. of days to maximum regression	Duration of remission (mos)
168	28	Actinomycin D + vincristine	21/30	PR	Infiltrative	Mixed	2	600	NR	—	—
173	41	None	—	—	Nodular	Uncertain	2	1020	CR	44	24+ (unmaintained)
178	46	Actinomycin D	20	PR	Florid	Monocellular	1	300	NR	—	—
183	30	None	—	—	Nodular	Mixed	5	1807	CR	169	13+ (unmaintained)
185	50	Actinomycin D	10	PR	Infiltrative	Mixed	6	1680	PR	135	5+ (maintained)
193	17	Actinomycin D	16.3	PR	Lymphadenopathic	Mixed	2	540	NR	—	—
		MOPP	1 course	NR							
217	42	Actinomycin D + vincristine	25/26	PR	Florid	Anaplastic	1	280	NR	—	—
229	35	Actinomycin D + vincristine	10/14	CR (3 mos)	Florid	Monocellular	2	600	NR	—	—
		Actinomycin D + vincristine	7.5/10.8	NR							
247	32	Actinomycin D + vincristine	18/24	PR	Infiltrative	Monocellular	1	300	NR	—	—
248	40	None	—	—	Nodular	Mixed	5	1750	PR	68	Lost to follow-up
249	70	None	—	—	Nodular	Monocellular	5	1450	CR	171	13+ (unmain-

249	70	None	—	—	Nodular	Monocellular	5	1450	CR	171	13+ (unmaintained)
254	45	Actinomycin D + vincristine	10/18	NR	Florid	Monocellular	4	1040	NR	—	—
390	45	Actinomycin D	9.75	PR	Florid	Mixed	1	300	NR	—	—

*CR = complete response; PR = partial response; NR = no response.
†Mechlorethamine, vincristine, prednisone, and procarbazine.

Bleomycin (table 2)

Six of ten patients (60%) treated with bleomycin had objective signs of tumor regression. In none, however, was the response complete and, in most, tumor regrowth was rapid. Among the objective responses, the three patients with florid tumors (Nos. 390, 463, and 467) each had a 50% reduction in the size of the easily measurable florid lesion, but patients No. 390 and No. 463 relapsed within 2 months. The third patient was lost to subsequent followup. Of the remaining three responding patients, one (No. 453) had extensive bone lesions and incapacitating pain. During the period of observation there was some sclerosis of previously destroyed bone with complete relief of pain for 3 months before relapse. A fifth patient (No. 151) had a large, deep-seated, anaplastic tumor of the thigh associated with profound edema. Complete resolution of edema occurred within 30 days of completion of the bleomycin treatment. The sixth responder (No. 373) had extensive nodular disease of one leg which was refractory to a combination of BCNU and actinomycin D. There was a 90% reduction in the number of nodules and no regrowth of new nodules over a 3-month period.

TOXIC EFFECTS

BCNU

The first five patients treated received the drug in 3-day courses at a dose level of 125 mg/m²/day as recommended by DeVita et al (7). At this dose level, severe toxic effects were encountered in three patients (Nos. 67, 88, and 173) whose white blood cell (wbc) counts fell to below 1500 cells/mm³ with associated thrombocytopenia. In one of these patients (No. 88) profound anemia (hemoglobin level, 4.6 g/100 ml) and fatal septicemia also developed. Patient No. 173 had severe pancytopenia which lasted for more than 4 months; 20 months after his last course of BCNU he still has a relative granulocytopenia. All subsequent patients received 200 mg/m² of BCNU in a single dose which was repeated every 6–8 weeks. Severe toxic effects did not develop in any of the patients although staphylococcal osteomyelitis developed in one patient (No. 124) who required prolonged antibiotic therapy and sequestrectomy while receiving the BCNU. Nausea and vomiting within 6 hours of drug administration was frequently seen but did not necessitate discontinuation of therapy.

Bleomycin

Hematologic toxic effects, as manifested by changes in hemoglobin concentration, total wbc, differential, and platelet counts, were not encountered. Hyperpigmentation, especially over bony prominences, was seen in nine patients, pruritis in five, cutaneous ulcerations in three, and alopecia in one. Occasionally, patients had some nausea and vomiting. The oral temperatures of all patients were taken hourly after each dose of bleomycin, and fever spikes, which usually occurred within 3 hours of drug administration, were seen in nine of ten patients. In most of the patients, the fever spikes decreased in severity as the course of treatment continued.

Only one patient (No. 168) received more than 300 mg of bleomycin and this patient died of rapidly progressive pulmonary fibrosis 2 weeks after completing a second full course of treatment. Weekly chest roentgenograms were taken of all patients who received bleomycin and pulmonary roentgenographic changes did not develop in any other patient.

DISCUSSION

Antineoplastic chemotherapy is the only modality of treatment routinely available for Kaposi's sarcoma in Uganda and in many other African countries where the disease is prevalent. Recently reviewed studies (2–4) have shown that this tumor is extremely sensitive to various chemotherapeutic agents, but especially to actinomycin D. Systematic evaluation of other agents, in patients whose tumors were refractory to actinomycin D, has established a high degree of sensitivity to NSC-45388(4) and now also to BCNU and bleomycin.

In the present study BCNU was used in four previously untreated patients with nodular disease and in 17 patients with more aggressive forms of Kaposi's sarcoma that had proved refractory to other forms of treatment.

TABLE 2.—Patient characteristics and responses to bleomycin

Patient No.	Age	Previous treatment	Dose level (mg)	Response*	Predominant clinical tumor type	Histologic type	Response to bleomycin	No. of days to maximum regression	Duration of remission (days)
151	45	See table 1			Florid	Anaplastic	PR	40	90+
168	30	See table 1			Infiltrative	Mixed	Died; pulmonary fibrosis	—	—
229	38	See table 1			Florid	Monocellular	NR	—	—
373	32	BCNU	810	NR	Nodular	Monocellular	PR	90	90+
		+							
		Actinomycin D	18.8						
390	45	See table 1			Florid	Mixed	PR	40	60
453	42	Actinomycin D	10	NR	Bone	Mixed	PR	40	60
454	36	Actinomycin D	3.5	NR	Florid	Monocellular	NR	—	—
455	76	Actinomycin D	7.5	NR	Aggressive nodular + pulmonary	Monocellular	NR	—	—
		+							
		Vincristine	12.0						
463	80	Actinomycin D	3.5	NR	Florid	Anaplastic	PR	40	Lost to followup
467	30	Actinomycin D	1.5	CR (2 mos)	Florid	Monocellular	PR	40	30
		Actinomycin D	10.0	CR (13 mos)					
		Vincristine	14.0	CR (5 mos)					
		NSC–45388	4000	NR					

*CR = Complete response; PR = partial response; NR = no response.

Although the four patients treated with BCNU as their first modality of therapy were not treated in a randomized trial, the response in each was gratifying. Controlled trials in a large series of patients with a followup period of several years would be necessary to establish the relative effectiveness of BCNU as opposed to other alkylating agents such as 2,3,5-tris(1-aziridinyl)-*p*-benzoquinone (8) in this indolent form of Kaposi's sarcoma.

The response rate of 29% in patients whose tumors were previously found to be refractory to actinomycin D is not as impressive as that produced by NSC-45388, but there is no question that BCNU is capable of providing good control of the disease for prolonged periods of time in selected patients. Especially gratifying is the unmaintained remission of 1 year in a young man with severe aggressive nodular disease with anaplastic histopathology that was refractory to actinomycin D and had never responded to MOPP combination chemotherapy. Likewise, excellent disease control has been maintained with BCNU in two patients with extensive nodular disease associated with deep tissue infiltration with tumor.

Bleomycin was only used in patients with refractory tumors and the overall objective response rate of 60% initially looks impressive. The durability of these responses, however, was disappointing with at least three of six responding patients relapsing within 2 months of discontinuation of the treatment. It appears clear that while bleomycin may ultimately be shown to play a role in the treatment of Kaposi's sarcoma it will probably be necessary to use it in combination with other effective agents. The inability of bleomycin to induce complete or durable remissions could be a dose- or schedule-related phenomenon; however, it has been shown in the present and previously published studies that the potential hazard of pulmonary fibrosis makes the use of higher doses inadvisable. Although it has been reported that other dose schedules offer no distinct advantage over the twice-a-week schedule in other tumors, it remains possible that other schedules of drug administration might ultimately prove more beneficial in Kaposi's sarcoma.

The current Uganda Cancer Institute protocol for Kaposi's sarcoma includes a randomized clinical trial of actinomycin D and vincristine with and without concomitant NSC-45388. In nonresponding patients or in patients whose diseases are refractory to primary therapy, phase II studies of adriamycin and vinblastine are planned. Future four-drug combinations worthy of consideration for clinical trials include bleomycin, actinomycin D, and vincristine with either NSC-45388 or BCNU. Kaposi's sarcoma also appears to be an excellent model for a controlled trial of intralesional bacillus Calmette-Guérin (BCG) therapy, and such studies in selected patients are also planned.

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LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Adriamycin: NSC-123127
BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-
Bleomycin: NSC-125066
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl) amino]tetrahydro-, 2-oxide, monohydrate

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride; HN2; nitrogen mustard

MOPP: combination chemotherapy including mechlorethamine, vincristine, prednisone, and procarbazine

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

2,3,5-Tris(1-aziridinyl)-*p*-benzoquinone: NSC-29215; CAS reg. No. 68-76-8; 2,3,5-tris(ethyleneimino)benzoquinone; triaziquone; Trenimon

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

5-Fluorouracil (NSC-19893) and Actinomycin D (NSC-3053) in the Treatment of Acute Leukemia in Children [1,2]

Vita J. Land, Paul G. Dyment, Diane Komp, G. Bennett Humphrey, Margaret P. Sullivan, and Kenneth A. Starling [3,4]

SUMMARY

Twenty-three children with late-stage acute leukemia in relapse were treated with 5-fluorouracil (5-FU) at a dose of 400 mg/m² twice weekly. No remissions were attained by 15 evaluable patients. Twenty-four additional children with acute leukemia in remission were treated with 600 mg/m² of 5-FU once weekly. The duration of remission maintenance with 5-FU following induction with other agents ranged from 14 to 190 days in 20 evaluable patients. Actinomycin D was also evaluated as a remission-maintenance agent in 24 children with advanced acute leukemia; the length of remission maintenance ranged from 21 to 155 days in 22 evaluable patients. Toxicity related to 5-FU administration was more pronounced in the twice weekly administration during attempted induction than in the weekly administration during remission maintenance. No severe toxicity occurred in patients who had remission maintenance with actinomycin D treatment. It was concluded that 5-FU was of no benefit in inducing remission, and neither 5-FU nor actinomycin D was of benefit in maintaining remission in those patients.

[Cancer Chemother Rep 57:335–339, 1973]

5-Fluorouracil (5-FU) has been shown to be effective in a wide range of animal tumor systems (1). The drug probably inhibits DNA synthesis via the competitive inhibition of thymidylate synthetase by its nucleotide derivative 5-fluoro-2′-deoxyuridine-5′-monophosphate, producing a thymidylic acid deficiency (2). 5-FU has been widely used in adult carcinomas at varying dose schedules and in a few children with various solid tumors (3, pp 195–226) and acute leukemia (4). Daily dosage schedules, although associated with evidence

[1] Received Mar 14, 1973; accepted May 24, 1973.

[2] 5-Fluorouracil: CAS reg. No. 51-21-8; 5-FU. 5-FU and actinomycin D were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute (NCI).

[3] This study was done by the Southwest Cancer Chemotherapy Study Group; Operations Office (CA-12644); Statistical Office (CA-12014). The following institutions participated in the study: Washington University School of Medicine, St. Louis, Mo (Vita J. Land, Abdel H. Ragab, Teresa J. Vietti; CA-05587); Cleveland Clinic, Ohio (Paul G. Dyment, Derrick Lonsdale; CA-04919); University of Virginia School of Medicine, Charlottesville (Diane Komp; CA-08223); University of Oklahoma School of Medicine, Oklahoma City (G. Bennett Humphrey, Daniel Lane; CA-11233); University of Texas, M.D. Anderson Hospital and Tumor Institute, Houston (Margaret P. Sullivan, Wataru W. Sutow; CA-03713); Baylor College of Medicine, Houston, Tex (Donald J. Fernbach, Kenneth A. Starling; CA-03161); University of Texas Medical School at San Antonio (Thomas E. Williams). Supported by Public Health Service research grants from the NCI, National Institutes of Health, Department of Health, Education, and Welfare.

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of antileukemic activity in four of 13 children resistant to conventional therapy, were associated with severe gastrointestinal and myelosuppressive toxic effects. This experience, as well as data indicating mild toxicity with a weekly schedule of administration of 5-FU (5), resulted in the Southwest Cancer Chemotherapy Study Group's decision to evaluate two schedules of 5-FU administration—twice weekly for remission induction and once weekly for remission maintenance.

Actinomycin D is an antibiotic whose antitumor effect has been confirmed in experimental tumors in animals (6) and in various solid tumors in man (3, pp 257–270; 7). Its mode of action is by binding the DNA, and thus selectively inhibiting DNA-dependent RNA synthesis (8). The current study, which tested the effectiveness of actinomycin D used as a single maintenance agent in acute leukemia of children, was prompted in part by a report (9) that suggested that the addition of actinomycin D and mechlorethamine [5] significantly prolonged remissions *maintained* by conventional therapy. This report was in contrast with other reports (10,11) indicating the lack of efficacy of actinomycin D therapy in *inducing* remissions.

[5] Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard.

METHODS

5-FU Given to Patients in Relapse

Twenty-three children who were under 18 years of age at the time of the study and had acute leukemia resistant to standard chemotherapeutic agents received 400 mg/m² of 5-FU twice weekly by intravenous (iv) injection (table 1). There were 14 boys and nine girls. Except for one child with acute myelogenous leukemia (AML), all children had acute lymphocytic leukemia (ALL) or acute undifferentiated leukemia (AUL). Patients were examined and had blood counts taken weekly during the 6-week trial of 5-FU. Bone marrow aspirations were performed every 2 weeks. National Criteria Committee criteria for evaluating chemotherapy in acute leukemia were used (12).

5-FU Given to Patients in Remission

Twenty-four children who were under 17 years of age and were in remission with acute leukemia were given 5-FU in an attempt to maintain their remissions. They received 600 mg/m² of 5-FU weekly, either orally or iv, until the onset of relapse as defined by standard criteria (12).

Table 2 summarizes details of the length and duration of past remissions, the total duration of disease, and the ages of 20 evaluable

TABLE 1.—5-FU given to patients in relapse

Patient No.	Age at start of 5-FU therapy (yrs)	No. of past remissions	Total duration of disease (days) from diagnosis to start of 5-FU induction	No. of doses of 5-FU received	Response
1	6 5/12	4	1332	8	None
2	9 2/12	5	704	8	None
3	12 9/12	3	461	12	None
4	5 0/12	6	1057	8	None
5	4 0/12	2	779	6	None
6	8 11/12	4	1809	8	None
7	8 11/12	4	1705	7	None
8	10 5/12	3	1703	10	None
9	6 5/12	4	945	9	None
10	10 10/12	5	1057	6	None
11	12 0/12	2	375	6	None
12	6 5/12	4	756	8	None
13	5 3/12	5	999	11	None
14	13 8/12	4	777	7	None
15	6 2/12	2	290	6	None

patients. There were 14 boys and six girls. All patients had ALL or AUL, except for one child with AML. Clinical and blood examinations were performed weekly, and bone marrow aspirations were performed at least every 4 weeks during this maintenance phase. Duration of remission was evaluated.

Actinomycin D Given to Patients in Remission

Twenty-two children (14 boys and eight girls, all with ALL or AUL) under 17 years of age who had acute leukemia in remission were given 500 $\mu g/m^2$ of actinomycin D weekly by iv injection until relapse occurred (table 3). The maximum individual dose was 500 μg. Physical examinations were performed and hematologic parameters and remission durations were measured at the same intervals as in patients receiving 5-FU maintenance therapy.

RESULTS

5-FU Given to Patients in Relapse

None of the 15 evaluable patients showed a decrease in the percentage of blast cells in the bone marrow or peripheral blood. Nine patients showed evidence of increasing numbers of blasts during the course of 5-FU therapy. Six patients, including the only one with AML, were inevaluable because of early death (3–19 days after 5-FU therapy was started), and two patients were inevaluable because of inadequate trial (fewer than six doses) due to toxicity. No toxic effects were noted in six of 15 patients. Some type of toxicity was noted in a total of nine patients. Bone marrow depression occurred in nine children (mild, three patients; severe, six patients). Moderate nausea and vomiting was reported in two patients. It was difficult in most cases to determine whether the bone marrow depression was due to marrow infiltration by leukemia or to the 5-FU therapy. Two children had both marrow and gastrointestinal toxic manifestations. All 23 patients died within 4 months after completing 5-FU treatment except for one patient (table 1, No. 11) who died 1 year later.

5-FU Given to Patients in Remission

As shown in table 2, the duration of 5-FU-

TABLE 2.—5-FU-maintained remissions

Patient No.	Age at start of 5-FU maintenance (yrs)	Total duration of disease (days) from diagnosis to start of 5-FU maintenance	Length of past remissions (days)						Length of 5-FU remission (days)
			1st	2nd	3rd	4th	5th	6th	
1	5 10/12	886	268	99	51	107	140	17	14
2	6 11/12	986	332	304	163				50†
3	16 3/12	2231	1904	160	78				190†
4	7 3/12	922	476	62	148				48
5	4 3/12	1019	534	65	37	86			27
6	8 10/12	1654	420	791	112	159			56
7	8 9/12	1304	773	112	43	36	81	64	88
8	7 7/12	1191	454	271	315				70
9	6 0/12	1326	392	227	80	210	96		22†
10	5 4/12	1592	329	31	706	175	155*		21
11	5 9/12	1341	539	273	120	257			30
12	7 3/12	417	112	46	110				84
13	4 5/12	301	154	58					25
14	5 1/12	600	353	81	54				29
15	7 1/12	1177	369	180	464				71
16	5 11/12	962	112	71	144	348	99		15
17	3 4/12	786	287	94	252				35†
18	8 5/12	615	245	83	147				29†
19	10 6/12	1417	1168	154					59†
20	16 2/12	750	181	290	141				28

*Actinomycin D-maintained remission.
†Subsequent remission maintained by actinomycin D.

TABLE 3.—Actinomycin D-maintained remissions

Patient No.	Age at start of actinomycin D maintenance (yrs)	Total duration of disease (days) from diagnosis to start of actinomycin D maintenance	Length of past remissions (days)							Length of actinomycin D remission (days)
			1st	2nd	3rd	4th	5th	6th	7th	
1	13 10/12	954	162	91	40	154	68	105		29
2	6 6/12	962	210	141	267	127				112
3	4 10/12	1401	329	31	706	175				115*
4	12 11/12	982	467	244	125					32
5	11 5/12	686	262	92	198					35
6	6 8/12	1875	972	147	161	76				70
7	4 5/12	545	140	56	35	91†				52
8	5 7/12	1240	478	110	313	62	44			21
9	8 11/12	943	342	244	235					77
10	6 11/12	1329	333	406	147	223				84
11	10 11/12	1996	753	518	106	185	105‡			28
12	7 4/12	1134	332	304	163	50§				22
13	3 5/12	608	175	36	197	56				28
14	8 0/12	728	210	35	73	208				56
15	8 1/12	535	243	152	46					23
16	16 10/12	2463	1904	160	78	190§				147
17	6 11/12	838	190	84	210	153				28
18	8 7/12	679	245	83	147	29§				28
19	6 7/12	1525	392	227	80	210	96	22§	118	21
20	3 7/12	856	287	94	252	35§				70
21	5 2/12	337	119	29						69
22	10 6/12	1510	1168	154	59§					57

*Subsequent remission maintained by 5-FU.

†Ninety-one days in remission after adriamycin (NCS-123127): not in relapse immediately before actinomycin D therapy.

‡5-FU failed to induce 5th remission.

§5-FU-maintained remission.

maintained remissions in 20 evaluable patients ranged from 14 to 190 days with a mean of 49.6 days and a median of 32.5 days. No toxic reactions were noted in 19 of the 20 evaluable patients. In the remaining patient, the platelet count decreased to a low of 31,000 platelets/mm^3. In another patient, severe vomiting induced by 5-FU necessitated a 26-day lapse in administration of the drug, followed by relapse of leukemia within 1 week. This was judged an inadequate trial due to toxicity. Three other children were inevaluable for remission maintenance because of protocol violations.

Actinomycin D Given to Patients in Remission

Twenty-two children were evaluable for remission maintenance after receiving actinomycin D. The duration of remissions maintained with actinomycin D in evaluable patients ranged from 21 to 155 days with a mean of 56.4 days and a median of 43.5 days. Two patients were not evaluable for remission maintenance because of protocol violations. There were no toxic reactions in 13 of the 22 evaluable patients. Moderate toxic reactions were noted in four patients (nausea and vomiting in two, granulocytopenia in one, and reactivation of erythema and alopecia in previously irradiated sites in one). Mild toxic effects were noted in five patients (granulocytopenia in one, thrombocytopenia in one, and nausea and vomiting in three).

DISCUSSION

The absence of a response among 15 patients who were evaluable for remission induction with 5-FU (table 1) indicates a response rate of less than 20% at 95% confidence limits (13). Although the median duration of an unmaintained remission late in the course of childhood

acute leukemia is not known, it would not be expected to be longer than the 33-56 days reported by others (14–17) for unmaintained (usually first and second) remissions. The data in both tables 2 and 3 indicate that in almost every case the duration of the 5-FU- or actinomycin D-maintained remission was shorter than the most recent past remission, indicating no obvious effectiveness of these drugs in prolonging remission.

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Phase I Study of Chromomycin A_3 (NSC-58514) [1,2,3]

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SUMMARY

Chromomycin A_3 was given to 26 patients with far-advanced cancer to ascertain the pattern of toxic effects of the drug when given in a 5-day course and to establish a dose schedule which would produce definite but clinically tolerable toxic effects. A total of 38 courses of treatment were given by rapid intravenous injection at dose levels which ranged from 0.25 to 1.1 mg/m²/day for 5 days. Most patients experienced no symptoms or toxic effects other than mild nausea, which usually occurred on the 1st day of treatment. After seven of 38 courses of treatment, there were transient increases in serum glutamic oxaloacetic transaminase and alkaline phosphatase values but there was no evidence of major hepatotoxicity. The drug was not associated with leukopenia and rarely caused thrombocytopenia. Extravasation of chromomycin A_3 into subcutaneous tissue caused pain, induration, and necrosis. At daily doses which exceeded 0.9 mg/m², significant impairment of renal function occurred in almost all patients. Acute renal tubular necrosis was documented in one patient who was given chromomycin A_3 at a dose level of 1.0 mg/m²/day for 5 days. Nephrotoxicity was not reduced when chromomycin A_3 was administered in a 5-hour infusion.

[Cancer Chemother Rep 57:341–347, 1973]

Chromomycin A_3 is an antibiotic isolated from cultures of *Streptomyces griseus* No. 7 (1). It has been shown to have antineoplastic activity in several model tumor systems (2–4) and has been reported to have activity against a number of solid tumors in humans (5,6).[7] The mechanism of action of chromomycin A_3 is believed to be related to its ability to inhibit DNA-dependent RNA polymerase and DNA polymerase (7–9). The interaction of chromomycin A_3 with DNA has been shown to be dependent on binding to guanine (10,11).

In the rat, the drug inhibits growth of the transplantable ascites tumors, Yoshida sarcoma and hepatoma AH130 (2,12). In mouse tumor systems, chromomycin A_3 inhibits Ehrlich ascites tumor, sarcoma 180, and lymphatic leukemia SN36 (2–4) but it has no significant

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[3] Chromomycin A_3: CAS reg. No. 7059-24-7; Toyomycin. Supplied by the Clinical Drug Distribution Section, Drug Development Branch, Drug Research and Development, DCT, NCI.

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[6] We wish to thank Dr. Claude Arnaud for the serum parathyroid hormone determinations.

[7] Slavik M, and Carter SK. Clinical brochure: chromomycin A_3 (NSC-58514) (Toyomycin). Prepared by the NCI, Bethesda, Md, 1972, 39 pp.

activity against L1210 leukemia.[8] In P388 leukemia, the cytotoxic effect of the drug is schedule-dependent and is greater than that of mithramycin.[8,9] Chromomycin A_3 is most effective against P388 leukemia at a dose level of 0.25 mg/kg/day for 5 days given intraperitoneally.[8]

Preclinical pharmacologic studies revealed the following values for the toxic effects of the drug when administered by rapid intravenous (iv) injection: in dogs the toxic dose low (TDL)[10] was 0.5 mg/m²/day for 5 days and the lethal dose (LD)[11] was 3.0 mg/m²/day for 5 days; in monkeys the TDL was 0.3 mg/m²/day for 5 days and the LD was 1.3 mg/m²/day for 5 days.[8] In rabbits, chromomycin A_3 is almost completely cleared from the plasma within 3 hours after a single iv injection of 0.5 or 1.0 mg/kg; the drug is eliminated in the urine and bile (13). Acute toxicity studies in dogs and monkeys revealed focal necrotic areas in lymphoid tissue, bone marrow, salivary glands, and the lamina propria of the large bowel (14). In one of seven dogs there were areas of acute renal tubular necrosis (14).

In the early 1960s, chromomycin A_3 had extensive clinical trials in Japan and South Africa involving more than 500 patients (5,6).[8] The majority of the patients in the Japanese and South African studies received doses of chromomycin A_3 varying from 0.5 to 1 mg daily for periods of 3–60 days.[8] The drug has also been administered on an intermittent schedule, most commonly, 0.5 mg twice a day biweekly.[8] Rates of objective response ranged from 35% in gastric and colorectal cancer and 45% in sarcomas of the bone and soft tissue to 73% in malignant lymphomas.[8]

Reported clinical toxic effects of chromomycin A_3 included nausea and vomiting associated with anorexia and weakness (particularly after daily doses of 1 mg or more), mild leukopenia, and necrosis at sites of infiltration. Despite its structural similarity to mithramycin, chromomycin A_3 was not reported to cause hepatic or renal toxicity, bleeding, or hypocalcemia.

The objectives of our study were to determine the pattern of toxic effects of chromomycin A_3 and to establish a dose schedule which produced definite but clinically tolerable toxic effects.

MATERIALS AND METHODS

Patients

All patients who were entered in this study had histologic confirmation of unresectable cancer for which no other means of therapy offered an expectation of cure or significant palliation. All patients were informed of the nature of their disease, of the investigational nature of the study, and of the limitations and risks attendant upon administration of the drug. We required that all patients be ambulatory, be maintaining a reasonable state of nutrition, and have an estimated lifespan greater than 1 month. Absolute contraindications to patient selection were a moribund state; frequent nausea and vomiting; extensive surgery, radiotherapy, or chemotherapy with cytotoxic agents within the preceding 30 days; leukopenia ($<$ 4,000 cells/mm³); thrombocytopenia ($<$ 150,000 platelets/mm³); or severe impairment of renal or hepatic function.

Of the 26 patients entered in this study, 19 had adenocarcinomas (18 of the gastrointestinal tract and one of the breast); three had squamous cell carcinomas of the tongue or esophagus; three had fibrosarcomas; and one had a leiomyoblastoma. Nine patients had had no previous chemotherapy. All patients were examined on the day before or the day of administration of chromomycin A_3. They were re-examined 3 and 6 weeks after treatment. The following studies were obtained on each patient prior to treatment, on the day following treatment, 3 weeks after treatment, and 6 weeks after treatment: hemoglobin, white blood cell (wbc) and differential count, platelet count, serum bilirubin, serum alkaline phosphatase, serum glutamic oxaloacetic transaminase

[8] *See* footnote 7.

[9] Mithramycin: NSC-24559; CAS reg. No. 18378-89-7.

[10] Toxic dose low (TDL): the lowest dose which produces drug-induced pathologic alterations in hematologic, chemical, clinical, or morphologic parameters; doubling this dose produces no lethality (*see* footnote 7).

[11] Lethal dose (LD): the lowest dose which produces drug-induced death in any animal during the treatment or observation period (*see* footnote 7).

(SGOT), serum calcium, prothrombin time, serum creatinine and 24-hour creatinine clearance, routine urinalysis, chest roentgenogram, and electrocardiogram.

Administration of Drug

Chromomycin A_3 was stored in powder form at 20 C. It was dissolved in sterile water at a concentration of 0.1 mg/ml immediately prior to administration and was given by rapid iv push into the tubing of an established infusion of 5% dextrose in water. For three patients, chromomycin A_3 was dissolved in 1000 ml of 5% dextrose in water in a light protected container and administered by iv infusion over a 5-hour period. All patients were seen by one of the investigators at least once daily. Temperatures, pretreatment and posttreatment pulses, and blood pressures were measured on each treatment day.

A course of treatment consisted of a daily injection for 5 consecutive days. We began our study at a daily dose of 0.5 mg/m² and increased the dose level in groups of three patients in increments of 0.1 mg/m². One patient became catastrophically ill after the three doses of chromomycin A_3 at 0.6 mg/m²/day, and until it became clear at a later postmortem examination that this episode was caused by an acute bacterial peritonitis, patients were entered at a daily dose level of only 0.25 mg/m². No patient was entered at a higher dose level until all patients who were treated at the previous level had been followed for at least 2 weeks.

All patients had wbc and platelet counts on Days 3 and 5 of treatment and twice weekly thereafter for the next 6 weeks. A serum creatinine or a plasma urea nitrogen level was determined weekly for 6 weeks. Patients were re-treated at 6-week intervals at the next higher dose level if they had shown no evidence of drug-induced toxicity and provided they had not had obvious progression of their disease with severe clinical deterioration. A total of 26 patients were treated by iv push; 16 patients received a single course, six patients received two courses, and four patients received three courses of therapy.

RESULTS

Toxicity

The incidence of gastrointestinal, hematologic, hepatic, and metabolic toxicity is shown in table 1. The majority of patients experienced nausea and a few vomited, usually only on the 1st day of treatment. These symptoms were mild in most patients and rarely required the administration of antiemetics. Hematologic toxicity was not clinically significant. No patient had a decrease in wbc to less than 4000 cells/mm³. There were five instances of thrombocytopenia that reached nadirs of 60,000–90,000 platelets from 12 to 42 days after initiation of therapy. There was no discernible correlation between the occurrence of thrombocytopenia and the dosage of chromomycin A_3.

Pain and induration developed in two patients at sites of extravasation of chromomycin

TABLE 1.—Toxicity with chromomycin A_3 therapy

Dose level (mg/m²/day × 5 days)	No. of courses	Toxic effects				
		Nausea and vomiting	Leukopenia (<4000 cells/mm³)	Thrombocytopenia (<100,000 platelets/mm³)	Increased SGOT level (>20 units)	Serum calcium level (<8.5 mg/100 ml)
0.25	3	0	0	0	0	0
0.5	3	1	0	1	0	0
0.6	8	2	0	1	2	1
0.7	3	2	0	0	1	0
0.8	7	4	0	1	1	1
0.9	6	4	0	2	0	0
1.0	5	5	0	0	0	2
1.1	3	3	0	0	3	3
Total	38	21	0	5	7	7

A_3. One of these patients had subsequent ulceration of the skin with complete healing by scar formation at 3 weeks.

Increases in SGOT and alkaline phosphatase levels, unaccompanied by changes in serum bilirubin levels, were noted after seven of 38 courses of treatment. Each of three patients treated at the highest dose level (1.1 mg/m²) had increases in SGOT greater than 20 international units (IU). Although all patients having a rise in SGOT had metastatic liver disease, in four, the concentrations of SGOT and alkaline phosphatase returned to pretreatment levels by 6 weeks suggesting hepatotoxicity secondary to chromomycin A_3 therapy.

Hypocalcemia occurred rarely at low doses but was noted in five of seven patients who were treated with 1.0 or 1.1 mg/m². Tetany was not observed although serum calcium concentrations declined to as low as 7.2 mg/100 ml. One patient who had a decrease in serum calcium concentration from 9.4 to 7.2 mg/100 ml had a concurrent decrease in the level of serum phosphate from 2.9 to 2.6 mg/100 ml, a decrease in renal tubular resorption of phosphate from 76% to 42%, and a decrease in urinary calcium from 30 to 10 mg/24 hours. This patient also showed a fourfold increase in the serum concentration of parathyroid hormone and had the most severe reduction in renal function among all patients studied.

Serum parathyroid hormone levels remained normal and appropriate for the levels of serum calcium in the other four patients who had quantified measurements of their renal function.

The majority of patients who received chromomycin A_3 at dose levels greater than 0.9 mg/m² showed significant impairment of renal function. As shown in table 2, seven of eight patients who received dose levels of 1 or 1.1 mg/m² had increases in serum creatinine levels to greater than 1.4 mg/100 ml. Proteinuria occurred after doses as low as 0.6 mg/m² and became increasingly frequent at higher doses. Although proteinuria developed in patients not showing a rise in the concentration of serum creatinine, three patients with abnormally elevated levels of serum creatinine did not have proteinuria. Hyaline casts and a few red and white blood cells were frequently seen in urine sediments after therapy with chromomycin A_3, but they were not directly related to the severity of impairment of renal function.

All patients in whom azotemia and proteinuria developed showed improvement at 3 weeks, and most had serum creatinine levels within the normal range at 6 weeks. Two of six patients who did not become azotemic after an initial course of chromomycin A_3 at 0.6 mg/m² had transient increases in serum creatinine concentrations after a second course at either 0.8 or 0.9 mg/m². Two patients received three courses at dose levels of 0.6, 0.9, and 0.9 mg/m² and showed no alterations in serum creatinine or urinary protein levels. One patient with no evidence of pre-existing renal disease had a transient increase in serum creatinine concentration from 0.9 to 1.4 mg/100 ml after a course of chromomycin A_3 at 0.5 mg/m². After a second course at 0.8 mg/m², his serum creatinine concentration rose from 0.9 to 2.8 mg/100 ml 2 days after treatment, and then fell to 1.6 mg/100 ml at 6 weeks where it has remained for 3 months.

TABLE 2.—Renal toxicity with chromomycin A_3 therapy

Dose level (mg/m²/day × 5 days)	No. of courses	Serum creatinine (≥1.5 mg/100 ml)	Urinary protein (>0.6 g/24 hrs)
0.25	3	0	0
0.5	3	0	0
0.6	8	0	2
0.7	3	1	1
0.8	7	2	2
0.9	6	0	2
1.0	5	5	4
1.1	3	2	3
Total	38	10	14

In order to define the nature and severity of renal impairment secondary to therapy with chromomycin A_3, the renal function of five patients was studied in greater detail. Standard renal clearance of inulin (C_{in}) to estimate glomerular filtration rate, and paraminohippurate (C_{pah}), to estimate effective renal plasma flow, was determined in five patients before treatment, 1 day after treatment (Day 6), and 5 weeks after treatment (Day 42; two patients only). These patients had normal pretreatment serum concentrations of creatinine and no history of renal disease. One patient was treated at a dose level of 0.6 mg, one at 0.8 mg, and three at 1.0 mg/m²/day for 5 days. As shown in table 3, patients who received the two lower doses had no alterations in either glomerular filtration rate (C_{in}) or effective renal plasma flow (C_{pah}). In contrast, in the three patients who received daily doses of 1.0 mg/m², significant impairment of renal function developed by the 6th day. Patient No. 3 had a 50% reduction in C_{in} and C_{pah}. Six weeks after treatment this patient still had impaired renal function; C_{in} and C_{pah} had increased to approximately 70% of pretreatment clearances. Patient No. 4 showed a 65% reduction in the clearance of inulin and paraminohippurate on the 6th day. A renal biopsy by light microscopy revealed no evidence of tubular injury and C_{in} and C_{pah} returned to pretreatment values by 6 weeks. Oliguria (ie, < 400 ml urine per day) did not develop in either patient. In patient No. 5, who had renal-function impairment on pretreatment clearances, acute renal failure developed with a profound reduction in the clearances of inulin and paraminohippurate on Day 6. This reduction was associated with oliguria, a rising serum creatinine concentration, and profound nausea and vomiting. Hospitalization was required to institute iv replacement and maintenance of water and electrolyte levels, and the patient experienced clinical and renal-function improvement. A renal biopsy on the 2nd day after treatment showed focal areas of acute tubular necrosis which involved the descending and ascending loops of Henle and the distal convoluted tubules (figs 1 and 2). There was loss of nuclei, disruption of the basement membrane of the tubular cells, sloughing of epithelial cells, active eosinophilic degenerative changes, and focal areas of regenerating tubular cells.

Although we could not be certain that the tubular damage caused by chromomycin A_3 is irreversible, we felt it was hazardous to give the drug by rapid iv injection at concentrations greater than 0.9 mg/m². We considered other methods of administration of chromomycin A_3 which might minimize its nephrotoxic effects. One possibility was to give the drug at lower doses over longer periods of time, and another possibility was to give the drug by slow infusion in an effort to avoid high concentrations reaching the renal tubules at any given moment. Since a 10-day schedule is under evaluation at another institution, we studied the toxicity of chromomycin A_3 given by slow infusion. The protocol was identical to the one described above except that the drug, instead of being given by rapid iv injection, was dissolved in 1000 ml of 5% dextrose and water and infused over a 5-hour period.

Three patients were treated by this slow infusion method. As shown in table 4, the first patient who was treated at a dose level of 0.5 mg/m²/day for 5 days showed an increase in the level of serum creatinine from 0.8 to 1.0

TABLE 3.—Effects of chromomycin A_3 therapy on renal function

Patient No., sex	Dose level (mg/m²/day × 5 days)	C_{in} (ml/min/1.73 m²)			C_{pah} (ml/min/1.73 m²)			Serum creatinine* (mg/100 ml)		
		Day 0	Day 6	Day 42	Day 0	Day 6	Day 42	Day 0	Day 6	Day 42
1, F	0.6	88	102	—	330	386	—	0.90	0.80	—
2, F	0.8	71	72	—	390	370	—	0.85	0.90	1.05
3, M	1.0	89	45	68	663	308	488	0.95	1.30	0.95
4, M	1.0	69	24	66	378	152	374	1.25	2.50	1.25
5, F	1.0	47	8	—	219	67	—	1.00	3.55	—

*Normal values: males = 0.8–1.2 mg/100 ml; females = 0.6–0.9 mg/100 ml (15).

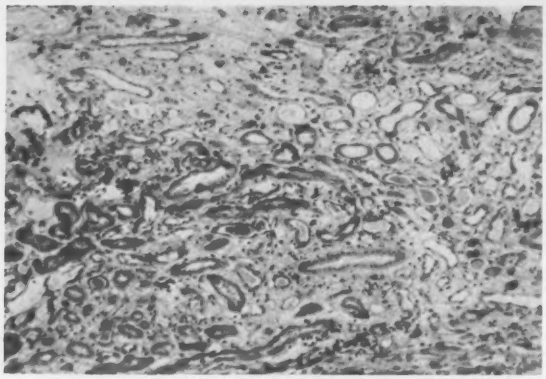

FIGURE 1.—Acute tubular necrosis secondary to chromomycin A_3 therapy. (× 100)

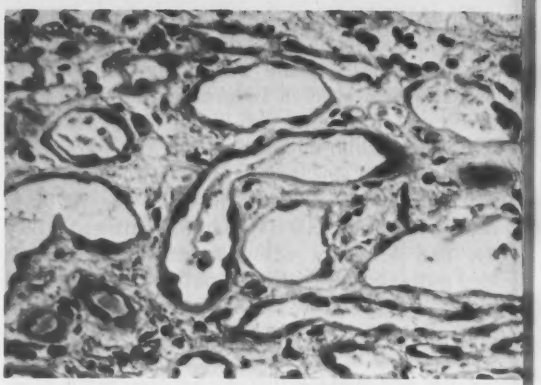

FIGURE 2.—Acute tubular necrosis secondary to chromomycin A_3 therapy. (× 400)

mg/100 ml by the 6th day. A second patient who received the same treatment had an increase in serum creatinine concentration from 1.5 to 1.8 mg/100 ml during the course of therapy, a sharp rise to 2.4 mg/100 ml 48 hours after the last dose, and a decline to 1.9 mg/100 ml by 3 weeks. A third patient, who was treated at a dose level of 0.75 mg/m², had a rise in serum creatinine concentration from 0.8 to 1.3 mg/100 ml during therapy, an increase to 1.8 mg/100 ml at the 6th day, and a decline to 1.3 mg/100 ml at 3 weeks. Rather than minimizing the renal effects of chromomycin A_3, the slow infusion method seemed to enhance the nephrotoxicity of the drug, and this approach was abandoned.

THERAPEUTIC RESPONSE

Although this study was not designed to assess therapeutic effect, we noted no evidence of objective response after the administration of chromomycin A_3 in 14 patients with measurable disease.

DISCUSSION

Chromomycin A_3, like other members of the family Actinomycetales, eg, streptomycin, neomycin, and kanamycin, is potentially nephrotoxic and damages primarily the renal tubular epithelial cells (16). The clinical effects may range from mild transient renal insufficiency to acute oliguric renal failure with morphologic evidence of acute tubular necrosis. There is an apparent dose-response relationship in that giving the drug by rapid iv injection in amounts greater than 0.9 mg/m²/day for 5 days consistently caused a rise in serum creatinine concentration to abnormal levels or decreased renal function as measured by standard renal clearances in patients studied in this series. In patients who had their renal function studied in greater detail, there was a 50% or greater reduction in glomerular filtration rate and effective renal plasma flow in those who received a dose of 1.0 mg/m²/day for 5 days.

We suggest that if chromomycin A_3 is to be

TABLE 4.—Changes in creatinine concentration with chromomycin A_3 by 5-hour infusion

Patient No.	Dose level (mg/m²/ day × 5 days)	Serum creatinine (mg/100 ml)						
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 6	Day 21
1	0.5	0.8	—	0.9	0.9	0.9	1.0	—
2	0.5	1.5	1.6	1.7	1.7	1.8	2.4	1.9
3	0.75	0.8	0.8	—	1.1	1.3	1.8	1.3

given by rapid iv injection, the dosage should not exceed 0.9 mg/m²/day for 5 days. This study did not determine if a high urinary output during the course of therapy would minimize the nephrotoxic effects of the drug. Impairment of renal function, however, did occur in well-hydrated patients. Certainly the drug should not be administered to any patient who is unable to take ordinary amounts of fluid by mouth, as evidenced by the patient in our series who sustained the most profound impairment of renal function and who additionally became severely dehydrated during chromomycin A_3 treatment.

Administration of the drug should be discontinued in all patients at the first appearance of an increase in the concentrations of serum creatinine or urinary protein. It should be noted, however, that significant impairment of renal function may occur without concomitant heavy proteinuria or abnormal elevation in the level of plasma urea nitrogen.

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Studies on Drug Resistance. V. Combination Chemotherapy With Cytosine Arabinoside (NSC-63878) Plus 6-Thioguanine (NSC-752) in Cytosine Arabinoside-Sensitive and -Resistant P815 Mouse Neoplasms [1,2,3]

Brief Reports and Preliminary Communications

W. Kreis [4,5]

The combination of cytosine arabinoside (ara-C) and 6-thioguanine, first used in the treatment of adult leukemia by Gee et al (1), is now a generally accepted combination in the chemotherapy for human acute leukemia (2–6). The synergistic action of the two compounds is believed to consist mainly in the reduction of 6-thioguanine toxicity by ara-C (7). It was also reported that in mice bearing the L1210 leukemia, bone marrow cells of the host can be protected selectively from 6-thioguanine toxicity by ara-C, whereas the leukemic cells still respond to 6-thioguanine treatment (8).

The aim of the present study was to determine whether it is reasonable to continue this combination after the cells become resistant to ara-C.

The system used was the ascitic form of the P815 neoplasm grown in BDF_1 mice. As demonstrated earlier (9), neither the ara-C-sensitive P815 cells nor the ara-C-resistant P815 cells (P815/ara-C) possess measurable amounts of ara-C deaminase. Furthermore, preliminary experiments demonstrated no significant influence of 6-thioguanine on deamination of ara-C by mouse kidney ara-C deaminase.

Groups of six BDF_1 mice were inoculated intraperitoneally (ip) with about 10^6 cells from either sensitive (P815) or resistant (P815/ara-C) neoplasms. After 24 hours, ip treatment was initiated and continued for 5 days. The drugs were dissolved in 5% carboxymethylcellulose. In the case of combinations, the two drugs were given simultaneously in the same syringe. The results of a representative experiment (table 1) indicate that the combination of ara-C (5 mg/kg) and 6-thioguanine (1 mg/kg) increased the median survival time (MST) of mice bearing ara-C-sensitive P815 neoplasms up to four times that of the controls. This finding confirmed the reports of Schmidt et al (7) for the L1210 tumor system. Either ara-C or 6-thioguanine given alone produced similar increases in MST, ie, 50%–88%. An increase in the dose of ara-C to 20 mg/kg in combination with 1 mg of 6-thioguanine did not increase the therapeutic efficacy but, rather, was less effective. An increase in 6-thioguanine to 5 mg/kg in combination with 20 mg/kg of ara-C (table 1) also did not improve the therapeutic efficacy and showed about the same effect in both sensitive and resistant lines.

In P815/ara-C neoplasms, 6-thioguanine at 1 and 5 mg/kg was as effective as in the parent line, whereas ara-C had no therapeutic effect at all, and the combinations of 6-thioguanine and ara-C showed no synergistic effect.

Toxicity, in the form of weight loss, was observed only in the groups receiving 6-thioguanine alone and was pronounced at the 5-mg/kg dose. Ara-C in combination with 6-thioguanine appears to reduce the toxicity of the latter drug, but this cannot be the only

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[3] Cytosine arabinoside: CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C.
6-Thioguanine: CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-.

[4] Memorial Sloan-Kettering Cancer Center, New York, NY.

[5] The author is indebted to Dr. Dorris J. Hutchison for continuous interest, Dr. Franz A. Schmid for providing the animals, and to Mr. Kevin Scully and Miss Joanne Salley for technical assistance.

TABLE 1.—Effectiveness of ara-C and 6-thioguanine combinations in sensitive (P815) and ara-C-resistant (P815/ara-C) neoplasms in BDF_1 mice

Treatment	Dose (mg/kg)	P815 MST (days)	P815 Change in MST (%)	P815 Weight change on Day 6 (g/mouse)	P815/ara-C MST (days)	P815/ara-C Change in MST (%)	P815/ara-C Weight change on Day 6 (g/mouse)
Control		8		+3.7	8		+3.3
Ara-C	5	12	+ 50	+2.3	8	0	+2.9
Ara-C	20	15(1)*	+ 88	+1.7	8	0	+1.8
6-Thioguanine	1	15	+ 88	−1.0	13	+62	−0.7
6-Thioguanine	5	13(1)*	+ 62	−2.7	13	+62	−3.3
6-Thioguanine	1	30(6)*	>272	−0.1	14	+75	+0.4
Ara-C	5						
6-Thioguanine	1	23	+188	0	14	+75	−0.6
Ara-C	20						
6-Thioguanine	5	14	+ 75	−0.3	14	+75	+0.2
Ara-C	20						

*Numbers in parentheses represent 30-day survivors.

reason for the synergistic effect of optimal drug combinations.

It is obvious in this experimental system that once the cells become resistant to ara-C, the combination of ara-C and 6-thioguanine does not increase the MST of the ara-C resistant line more than 6-thioguanine alone. As reported previously (10), the only biochemical difference so far observed between the sensitive and resistant lines is the lack of a deoxycytidine-ara-C kinase.

Enough accumulated evidence indicates that the phosphorylation of ara-C to its 5′-diphosphates and 5′-triphosphates is essential for its cytotoxic activity in experimental (9,11–15) as well as clinical (16,17) studies. Ara-C deaminases, such as those reported in patients whose leukemias were resistant to ara-C (17–19), probably cannot influence the synergism of ara-C and 6-thioguanine, since our experimental systems, either sensitive or resistant to ara-C, have no detectable amounts of this ara-C-inactivating enzyme. The biochemical mechanism for this synergism is presently under investigation.

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Treatment In Vivo of R3230AC Carcinoma of the Rat With Estradiol Mustard (NSC-112259) of Its Molecular Components [1,2,3]

Brief Reports and Preliminary Communications

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Estradiol mustard, a steroidal alkylating agent comprised of an estradiol-17β nucleus with two cytotoxic chlorphenacyl side groups, was synthesized and described by Wall and coworkers (1). Estradiol mustard is therapeutically active in several rodent tumors and possesses biologic characteristics of both an estrogen and an alkylating agent (1,2). It is currently undergoing clinical trials by the Eastern Cooperative Oncology Group.

In this study we compared the effects of estradiol mustard with those of its estrogen and alkylating moieties (administered separately or in combination) in female Fischer 344 rats with transplanted R3230AC mammary adenocarcinoma. This tumor is responsive to estrogen although it does not regress after ovariectomy of the host (3). However, specific estrogen-binding proteins have been reported in the tumor grown in intact or ovariectomized hosts (4).

MATERIALS AND METHODS

Rats weighing 120–140 g received tumor transplants by sterile trocar technique. The animals were then divided into seven treatment groups with seven animals in each group. The phenester [8] and estradiol mustard were dissolved in an appropriate volume of sesame oil (Beacon); the estradiol valerate [9] was prepared in sesame oil by the manufacturer. Each animal was initially given a single subcutaneous injection of test compound in 1 ml of sesame oil or vehicle alone and, subsequently, two weekly injections for a total of 21 days. Each injection was three and one-half times the daily dose listed in table 1.

The doses of estradiol valerate and estradiol mustard for treatment groups C and E have been reported to inhibit several mammary tumors including R3230AC (2,5). Treatment for groups B and D was administered orally in equitoxic (R. Hilf, personal communication) doses, as was treatment for group F, on the assumption that there is no additive toxicity when estradiol valerate and phenester are combined. Animals in groups D and G received equimolar amounts of estradiol mustard (group

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[2] Supported in part by American Cancer Society Institutional Research Grant IN-18L and grant CA-11198 from the National Cancer Institute (NCI), National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

[3] Estradiol mustard: CAS reg. No. 22966-79-6; estra-1,3,5(10)-triene-3,17β-diol, bis[[*p*-[bis(2-chloroethyl)-amino]phenyl]acetate]. Estradiol mustard (Upjohn Co., lot No. 9306-CYP-50B) and phenester (Upjohn Co., lot No. 9306-CYP-32A; reported to contain no free estradiol) were obtained through the courtesy of Dr. D. Jane Taylor, Breast Cancer Program Coordinating Branch (formerly Endocrine Evaluation Branch), Division of Cancer Biology and Diagnosis, NCI. Estradiol valerate was obtained from E. R. Squibb & Sons, Inc., New Brunswick, NJ.

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[8] Phenester: NSC-116785; CAS reg. No. 31386-35-3; acetic acid, [*p*-[bis(2-chloroethyl)amino]phenyl]-, ethyl ester.

[9] Estradiol valerate: NSC-17590; CAS reg. No. 979-32-8; estra-1,3,5(10)-triene-3,17β-diol, 17-valerate.

D) or its components, estradiol-17β and phenester (group G).

Rat weights were recorded on Days 1, 10, and 18; on Day 21 all animals were killed and necropsies were performed. Carcass, tumor, and uterine weights were recorded.

RESULTS

Results are shown in table 1. All animals survived except for four of the seven in group G (high doses of estradiol valerate plus phenester). In most groups a modest increase in carcass weight comparable to that of the control group was noted. Rats treated with estradiol valerate or estradiol valerate plus phenester had little increase in carcass weight, perhaps because of drug toxicity. Uterotropic effects were seen in all groups given estradiol valerate; they were less pronounced in the groups treated with estradiol mustard alone. The group treated with phenester did not exhibit an increase in uterine weight. Administration of drug combinations, ie, estradiol valerate plus phenester, produced the greatest inhibition of tumor growth. Treatment with estradiol mustard alone resulted in a significant inhibition of tumor growth within 21 days. Separate administration of the moieties of estradiol mustard resulted in only a small decrease in the size of the tumors after 21 days.

DISCUSSION

The uterotropic response to estradiol valerate reported in this paper is similar to or slightly greater than that reported by Hilf and co-workers (5), but the therapeutic effect is less, ie, the ratio of tumor weight in treated animals to tumor weight in control animals was 0.50 in their report and 0.82 in our study. In addition, they noted a substantial difference between the carcass weights of rats treated with estradiol valerate and the carcass weights of control rats. The modest difference observed in our experiments is probably insignificant. The changes in body and uterine weights seen after the administration of estradiol mustard do not substantially differ from those noted by Vollmer and his colleagues (2), but the antitumor effect is somewhat less.

Estradiol mustard had considerably less antitumor effect and toxicity than did an equimolar amount of its two molecular constituents (group D versus group G). These differences in carcass and tumor weights may suggest that combination therapy is more effective than therapy with estradiol mustard alone. However, admin-

TABLE 1.—Effects of treatment in vivo of R3230AC mammary carcinoma of the rat with estradiol mustard or its molecular components

Group	Drug	Dose* Mg/kg/day	Dose* μMol/kg/day	Survivors	Body weight difference (g)	Uterine weight † (mg)	Tumor weight † (g)	Tumor weight T/C ‡
A	None	—	—	7/7	+18	274 ± 22	1.25 ± 0.22	—
B	Phenester	2.0	7.27	7/7	+19	253 ± 20	0.79 ± 0.27	0.63
C	Estradiol valerate	1.0	2.78	7/7	+ 7	807 ± 33	1.03 ± 0.19	0.82
D	Estradiol mustard	5.0	6.35	7/7	+22	451 ± 32	0.71 ± 0.22	0.57
E	Estradiol mustard	10.0	12.69	7/7	+16	384 ± 10	0.58 ± 0.10	0.46
F	Estradiol valerate + Phenester	1.0 2.0	2.78 7.27	7/7	+ 3	707 ± 42	0.16 ± 0.05	0.13
G	Estradiol valerate + Phenester	2.3 3.5	6.35 12.69	3/7	− 7	750 ± 41	0.07 ± 0.02	0.06

*The total dose calculated on a per-day basis was then given twice weekly.
†All data represent the mean ±SE for all surviving animals in that group as recorded on Day 21.
‡T/C = weight of tumor in treated group/weight of tumor in control group.

istration of higher doses of estradiol mustard would be necessary to determine whether its therapeutic effect can equal or exceed those seen for the combination of estrogen and alkylating agent. The less profound uterotropic effect of estradiol mustard as compared with an equimolar amount of estradiol valerate or estradiol valerate plus phenester suggests that metabolism or hydrolysis in vivo of estradiol mustard to free estradiol did not occur.

The estrogen and alkylating agent given together as separate entities were most effective in this study. Treatment with the combination was accompanied by the greatest mortality. The inhibition of tumor growth seen in animals from group F was not additive. The administration of phenester and estradiol valerate separately in amounts equal to those for the combination therapy (group F) caused losses in tumor weight not equal to those for the combination (T/C of 0.62 [group B] × 0.82 [group C] = 0.51 versus T/C of 0.13 [group F]). These data suggest that the antitumor effects of the drugs are synergistic. This synergism might result from increases in tumor sensitivity to the cytotoxic agent caused by estradiol (2).

Few previous reports of estrogen plus alkylating agent combination therapy are available. In postmenopausal metastatic carcinoma of the breast in humans, combination therapy with cyclophosphamide and diethylstilbestrol had an additive rather than a synergistic effect (6).[10] In estrogen-dependent mammary tumors induced by 7,12-dimethylbenz[*a*]anthracene,[11] administration of cyclophosphamide inhibited both tumor stimulation by estrogen and tumor response to high doses of estrogen (7). Our results suggest that estrogen and alkylating agent combinations may have positive synergism in the treatment of mammary carcinoma. Double agents such as estradiol mustard containing fixed proportions may be less flexible than single-drug combinations. This possibility, as well as the elucidation of the mechanism of action of estradiol mustard, deserves further investigation.

[10] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

Diethylstilbestrol: NSC-3070; 4,4′-stilbenediol, *α,α′*-diethyl-.

[11] 7,12-Dimethylbenz[*a*]anthracene: NSC-408823; CAS reg. No. 57-97-6; DMBA.

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Phase II Study of *Cis*-diamminedichloroplatinum (NSC-119875) in Advanced Carcinoma of the Large Bowel [1,2,3]

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Cis-diamminedichloroplatinum, an agent with antineoplastic activity in a variety of animal tumor screens (1), has been reported in phase I studies to show effectiveness in the treatment of some human cancers (2–6). This drug, however, has not had extensive trial in phase II studies.

In phase I studies, the major toxic effects associated with *cis*-diamminedichloroplatinum therapy were nausea and vomiting followed by prolonged periods of anorexia, dose-dependent impairment of renal function, leukopenia and thrombocytopenia which occurred as late as 4 weeks after treatment, hyperuricemia, and impairment of hearing primarily in the high-frequency range. The dose-limiting toxic effect was renal toxicity which occurred in approximately 25% of the patients who received 50 mg/m^2 and in 61% of the patients who received 100 mg/m^2 in a single dose (6). Our study was designed to look for the therapeutic activity of *cis*-diamminedichloroplatinum at a single dose level of 50 mg/m^2 and to compare, in a controlled manner, the rate of therapeutic response achieved with *cis*-diamminedichloroplatinum to that achieved with 5-fluorouracil (5-FU).[6]

MATERIALS AND METHODS

Patient Selection

All patients who were studied had histologic confirmation of unresectable cancer of the colon or rectum. They were informed of the nature of their disease, of the investigational nature of this study, and of the risks and limitations of treatment with this drug. All patients were ambulatory, were maintaining a reasonable state of nutrition, and had an estimated lifespan of greater than 2 months. Contraindications to entry into this study were severe debilitation; frequent nausea and vomiting; surgery, radiotherapy, or chemotherapy with cytotoxic agents within the previous month; leukopenia (<4000 cells/mm^3); thrombocytopenia (<150,000 platelets/mm^3); impaired hearing; or impairment of renal function (serum creatinine concentration > 1.2 mg).

Twenty-nine patients who had not received previous chemotherapy were randomly assigned to *cis*-diamminedichloroplatinum or 5-FU. 5-FU was administered in a 5-day course either by rapid intravenous (iv) injection at a dose level of 13.5 mg/kg/day or orally at a dose level of 20 mg/kg/day. Those patients who showed progression while receiving *cis*-diamminedichloroplatinum were crossed over to 5-FU and those receiving 5-FU were crossed over to *cis*-diamminedichloroplatinum if they were still suitable treatment candidates. In addition, 12 patients who had had previous chemotherapy were entered directly into the study with *cis*-diamminedichloroplatinum.

The clinical features of the 32 patients (16 men and 16 women) chosen for *cis*-diamminedichloroplatinum therapy were as follows. Eighteen patients had had previous therapy with cytotoxic drugs: 14 patients, 5-FU; five, methyl-CCNU; five, isophosphamide; one, cy-

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[3] *Cis*-diamminedichloroplatinum: CAS reg. No. 1853-33-9; CACP. Supplied by the Clinical Drug Distribution Section, Drug Development Branch, Drug Research and Development, DCT, NCI.

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[6] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

clophosphamide; and one, NSC-82196.[7] Fourteen patients had received no prior chemotherapy. Six patients had received prior radiation therapy. The patients ranged in age from 28 to 73 years (mean, 56 years).

Treatment Methodology

Cis-diamminedichloroplatinum was stored as a powder in 10-mg vials and was dissolved in water at a concentration of 1 mg/ml immediately prior to administration by rapid iv push. A course of treatment consisted of a single injection at a dose of 50 mg/m^2.

Each patient was examined on the day prior to or on the day of administration of *cis*-diamminedichloroplatinum. After therapy, leukocyte counts were obtained twice a week; platelet counts and blood urea nitrogen levels or serum creatinine values were obtained once a week for 5 weeks. All patients were re-examined at 5 weeks after treatment. Those patients who remained objectively stable or showed improvement were re-treated with *cis*-diamminedichloroplatinum at the same dose level as the initial treatment provided there had been no evidence of toxicity. Patients who showed impairment of renal function or significant impairment of hearing were not re-treated.

RESULTS

Toxicity

The toxic reactions that occurred after the first course of therapy with *cis*-diamminedichloroplatinum are shown in table 1. Almost every patient had repetitive vomiting or retching which started from 1½ to 4½ hours after the drug was administered and lasted from a few minutes to 7 hours. The majority of patients had no gastrointestinal symptoms after therapy but two patients experienced persistent anorexia for 1 week after therapy. Eight of 28 adequately evaluated patients who had had no known pre-existing renal disease experienced impairment of renal function after their first course of *cis*-diamminedichloroplatinum therapy as evidenced by increases in the concentration of serum creatinine or in the blood urea nitrogen level and decreases in creatinine clearance. These abnormalities in renal function were mild and reversible. Five patients who received two courses of *cis*-diamminedichloroplatinum, one who received three courses, and one who received four courses showed no alteration in renal function. One patient had a rise in serum uric acid from 4.2 to 7.9 mg/100 ml and one had a rise from 10.6 to 13 mg/100 ml. Both rises were noted 5 weeks after therapy and both coincided with impaired renal function. A mild decrease in auditory acuity above 4000 hertz was demonstrated by audiometric examination in eight patients. Only one of these patients noted a decrease in ability to hear normal conversational tones. In patients who received multiple courses, audiometric abnormalities were progressively more pronounced with each course.

TABLE 1.—Toxic reactions to first course of *cis*-diamminedichloroplatinum treatment in 32 patients

Toxic reaction	Observed frequency*
Nausea	32/32
Vomiting	31/32
Hearing loss	
subclinical	8/26†
clinical	1/26
Renal-function impairment	6/28
Leukopenia	
< 4000 cells/mm^3	4/31
< 2000 cells/mm^3	—
Thrombocytopenia	
< 150,000 platelets/mm^3	8/31
< 100,000 platelets/mm^3	2/31
< 50,000 platelets/mm^3	—
Anemia (fall in hemoglobin level > 2.0 g/100 ml)	2/28

*No. of patients with toxic reactions/No. of patients evaluable.

†Evaluations in this and subsequent categories completed with fewer than 32 patients because of non-drug-related death or severe clinical deterioration in the remaining patients.

[7] Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-.

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino] tetrahydro-, 2-oxide, monohydrate.

NSC-82196: CAS reg. No. 5034-77-5; imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]-.

Hematologic toxicity was insignificant. Only three of 31 patients had decreases in white blood cell counts to less than 4000 cells/mm³, and none had a decrease to less than 3000 cells/mm³. The nadirs occurred at Days 37, 34, and 18. Eight patients had decreases in platelet counts to less than 150,000 platelets/mm³; two of these were to less than 100,000 platelets/mm³ (80,000 and 88,000 cells/mm³). The nadirs of thrombocytopenia were quite variable and ranged from Day 4 to Day 32 (median, Day 15). Significant anemia developed in two patients (fall in hemoglobin level > 2 g/100 ml).

Therapeutic Response

Among the 32 patients treated with *cis*-diamminedichloroplatinum, 25 showed definite progression at 5 weeks and were not re-treated. Five remained objectively stable and received a second course of therapy at 5 weeks. Four of these patients showed progression at 10 weeks, and one has remained stable at 15 weeks after three courses of therapy. Two patients showed mixed responses. One patient with multiple pulmonary metastases showed regression of some lesions and progression of others. One patient showed significant regression of hepatomegaly with improvement of liver-function tests, but during this same time a new and progressing subcutaneous metastasis at the liver biopsy site developed.

In the controlled crossover comparison of *cis*-diamminedichloroplatinum and 5-FU, four of 23 patients (17.5%) who were treated with 5-FU showed an objective (> 50%) response which was maintained for 10 weeks after the initiation of therapy. An additional three patients showed objective responses at 5 weeks but progression at 10 weeks. No objective responses were noted among the 19 patients treated with *cis*-diamminedichloroplatinum.

DISCUSSION

At the dose level used in this study, *cis*-diamminedichloroplatinum appears to have little value in the therapy of colorectal adenocarcinoma, and it is significantly inferior to 5-FU in therapeutic activity. The low hematologic toxicity of this agent may allow it to be used simultaneously with other drugs which do depress bone marrow function. The negative results in this study should not mitigate against the evaluation of *cis*-diamminedichloroplatinum in combination regimens.

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High-Dose Intermittent Intravenous Infusion of Procarbazine (NSC-77213) [1,2]

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Brief Reports and Preliminary Communications

Procarbazine has become an important agent in the treatment of lymphomas (1). This agent is usually given in daily oral doses of 100–200 mg/m², although previous studies have demonstrated the clinical activity of this agent given by the intravenous (iv) route (2–4).

The increased efficacy of other cancer chemotherapeutic agents, such as methotrexate (5) and cyclophosphamide (6),[4] when given in intermittent high-dose parenteral schedules as compared to daily oral therapy, has prompted us to re-examine the use of procarbazine by high-dose intermittent iv infusion. The purpose of this study was to delineate the single highest tolerated dose of procarbazine which could be administered on an intermittent basis.

STUDY DESIGN AND PATIENT SELECTION

Procarbazine in crystalline form was obtained from Hoffmann-LaRoche in sterile vials and was reconstituted immediately prior to use by the addition of 25–50 ml of D5W. The appropriate dose was then administered iv over a 10–15-minute period.

Seventeen patients with metastatic malignancy refractory to standard therapy were included in the study. Trials were conducted at six different dose levels, beginning with 300 mg/m² and reaching a peak of 2500 mg/m² (table 1). Between two and four patients were entered in the study at each new level; if that dose level proved safe and was well tolerated in a minimum of two patients, the trial dose was escalated by 300–500 mg/m² in subsequent patients. No individual patient received procarbazine at two different dose levels, and if a patient received multiple doses of procarbazine, the first and second doses were separated by 2 weeks to allow any bone marrow suppression from the first dose to become apparent. Thereafter, repeat doses were given at weekly intervals.

RESULTS

Of the 17 patients treated iv with procarbazine, 11 received multiple courses of treatment at a single dose level while six received only a single dose. The major dose-limiting toxicity associated with the therapy was neurologic (table 1). Mild lethargy was noted by one of two patients receiving 300 mg/m², while somnolence during the period of infusion was seen in one of two patients treated with 600 mg/m². At 1000 mg/m², somnolence lasting 4–6 hours was observed in two of three patients, followed by lethargy during the recovery period. These patients were able to return to a normal level of function within 24 hours. At doses of 1500 mg/m² or greater, seven of ten patients experienced prolonged periods of somnolence lasting up to 72 hours followed by a recovery period characterized by restlessness, apprehension, and emotional lability. Numbness and weakness of lower extremities were also noted by three patients, and bothersome paresthesias were noted by two patients. Two patients who received 1500 and 2000 mg/m² respectively became disoriented immediately after therapy and remained confused and somnolent for 1–3 days. Nausea and vomiting, which was noted in all patients, was more prolonged in those patients treated with more than 1000 mg/m².

[1] Received June 18, 1973; accepted June 21, 1973.

[2] Procarbazine: CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride.

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[4] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

TABLE 1.—Toxic effects in patients treated with high-dose intermittent iv doses of procarbazine

Dose (mg/m²)	No. of patients	No. of patients with— Nausea and vomiting: Mild	Nausea and vomiting: Severe	Neurotoxicity: Lethargy	Neurotoxicity: Somnolence	Neurotoxicity: Other	Myelosuppression: Wbc nadir 2–4 × 10³ cells	Myelosuppression: Wbc nadir <2 × 10³ cells	Myelosuppression: Platelet nadir 50–100 × 10³ cells	Myelosuppression: Platelet nadir <50 × 10³ cells
300	2	0	2	1	0	Diarrhea (1)	0	0	0	0
600	2	0	1	0	1	—	0	0	0	0
1000	3	1	2	2	2	Dizziness (1)	1	1 (after 2nd dose)	0	0
1500	4	0	4	1	3	Disorientation (1) Dizziness (2) Weakness of extremities (1)	0	0	0*	0*
2000	4	0	4	1	2	Dizziness (1) Peripheral neuropathy (1) Disorientation (1)	2	1 (after 3rd dose)	1	1 (3rd dose)
2500	2	0	2	2	2	—	1	0	0	0

*No change in counts in 1 patient with blastic crisis of chronic myelogenous leukemia.

While myelosuppression has been the major toxic effect associated with orally administered procarbazine, serious depression of peripheral blood counts was not observed with any of the initial doses of iv administered procarbazine at any of the dose levels used in this study. Three patients who received 1000, 2000, and 2500 mg/m² respectively, had a transient decline in their white blood cell counts to 3000–4000 cells/mm³ after the first dose, while one patient who received 2000 mg/m² experienced a 4-week period of moderate leukopenia (nadir, 2000 cells/mm³) and thrombocytopenia (nadir, 72,000 platelets/mm³). However, myelosuppression appeared to be cumulative in that four of six patients who received multiple weekly doses of procarbazine at a dose level of 1000 mg/m² or greater had a progressive decrease in peripheral counts (table 1).

There were no objective antitumor responses.

DISCUSSION

Although procarbazine has been in clinical usage since 1963, the iv use of this agent has received little attention to date despite promising activity by this route in earlier studies (2,3). In the present study it appeared that intermittent iv doses of 300–1000 mg/m² can be given safely on an outpatient basis in patients with normal hepatic function, although somnolence, nausea, and vomiting should be anticipated during the 24 hours after the infusion. Doses above 1000 mg/m² regularly produced prolonged neurotoxicity which would limit the use of procarbazine at this dose level to intermittent infusions given in a hospital setting. The severe neurotoxicity observed in a patient with hepatic metastases who received 1500 mg/m² suggests that doses at this level or above should be used with extreme caution in patients with extensive liver involvement.

The reasons for neurotoxicity observed in these studies are not clear, but this toxicity clearly mimics the hypnotic and convulsant properties of the drug in rodents. Procarbazine may affect neurotransmittor metabolism, either as an inhibitor of monoamine oxidase (7) or through depletion of the co-factor, pyridoxal phosphate (8). The ability of pyridoxine to reverse or prevent neurotoxicity was not ex-

plored in this study, but the use of pyridoxine should be undertaken with caution in view of the potentiating effect of the co-factor on procarbazine-induced convulsions in mice (8).

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Antitumor Activity of a Homo-aza-steroidal Ester of [*p*-[Bis(2-chloroethyl)amino]phenyl]acetic Acid (NSC-71964) [1,2]

Brief Reports and Preliminary Communications

P. Catsoulacos [3] and L. Boutis [4]

Steroidal esters of [*p*-[bis(2-chloroethyl)-amino]phenyl]acetic acid, such as phenesterin,[5] are known for their antineoplastic action (1–3) and are less toxic than some of the well-known nitrogen mustards in general use. In pursuing our interest in the synthesis of steroidal-like alkylating agents, we have chosen homo-aza-steroids as carriers of nitrogen mustard, and especially the 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam, which, by interacting with the [*p*-[bis(2-chloroethyl)amino]phenyl]acetic chloride, gave the 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-[*p*-[bis(2-chloroethyl)amino]phenyl]acetate (4). The following are the properties of the substance: m.p. 131–132° (acetone-*n*-hexane); 65% yield; ν max 3170, 3050 (NH), 1725 ((CO)O–), 1675, 1650 (NH(CO)–); nmr showed at τ 6.52 (–CH_2CO–), 6.3 (–N(CH_2CH_2–)$_2$), and 5.25 (C_3–H) in addition to the common peaks; mass spectrum, M$^+$ 562; insoluble in water.

In this paper, we are reporting our studies on the antineoplastic action of the ester on three experimental tumors.

MATERIAL AND METHODS

The compound was tested against T8 Guérin tumor in Wistar rats, B16 melanoma in C57 black mice, and Theagenion-Bahner (Th-B) angiosarcoma in C3Hb mice. The latter transplantable tumor is of chemical origin and appeared in C3Hb mice after they were fed 1-(4-dimethylaminobenzylidene)indene (5) for some months. The average survival of the Th-B angiosarcoma-bearing mice is about 4 weeks. All tumors were injected subcutaneously with an acceptance rate of approximately 100%.

The substance was used as a 5-mg/ml suspension in corn oil and was given in dose schedules that were selected after a pilot study showed that the substance was active. The LD50 over 30 days was estimated in this study and was found to be about 70 mg/kg for rats and 58 mg/kg for mice after intraperitoneal (ip) injection.

Clinical observations on the response of the animals to the drug were done daily and the volume of the tumor was estimated at 5-day intervals. Estimation of the tumor volume was calculated from the formula

$$V = \frac{\text{length} \times \text{width} \times \text{depth}}{2}.$$

The T/C relation was estimated on the 10th day in experiments involving newly transplanted tumors and on the 20th day in experiments involving established tumors. Animals that died during the experiment were examined thoroughly for macroscopic and histologic alterations of their organs. Separate studies were done to establish alterations in the blood, the bone marrow, and the spleen by killing groups of animals 24 hours, 48 hours, 5 days, 15 days, or 25 days after ip injection of the compound.

The most important criteria for assessing the effectiveness of the compound are the inhibition of the tumor's growth and the survival of the animals. Disappearance of the tumor followed by tumor-free survival for more

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[2] NSC-71964: CAS reg. No. 10477-72-2.

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[5] Phenesterin: NSC-104469; CAS reg. No. 3546-10-9; cholesterol, [*p*-[bis(2-chloroethyl)amino]phenyl]-, acetate.

than 3 months was considered to constitute a cure.

For better evaluation of the mode and degree of antineoplastic action, additional experiments were done under comparative conditions with cyclophosphamide [6] and with the homo-aza-steroid at doses of 5 mg/ml in corn oil. Some representative results are also included in this study.

RESULTS

The data collected in this study are summarized in tables 1–3 which show the excellent activity of the compound in T8 tumor and the Th-B angiosarcoma. Very little activity was noted in the B16 melanoma. In animals with established tumors the therapeutic effect on the T8 tumor and the Th-B angiosarcoma was evident from the disappearance of the tumor which became necrotic, showed some bizarre cells, and was finally replaced by a giant cell foreign body reaction with calcium deposits.

TOXICITY

Death of rats and mice after toxic doses of the ester usually occurred from gastrointestinal bleeding between the 5th and 12th day after ip injection. After ip doses of 100 mg/kg or more, convulsions and death occurred in 10–24 hours.

The major toxic effect was noted 4–7 days after the administration of therapeutic doses of the compound and was characterized by depletion of the cellular elements of the bone marrow and the spleen. Twenty-four hours after the administration of a single dose of 25 mg/kg, a slight leukopenia due to diminution of lymphocytes was detected, while 5 days after the same dose a severe leukopenia was found with depletion of the erythroid series in the bone marrow and maturation arrest of granulopoiesis. Five days after a single dose of 50 mg/kg, the bone marrow was aplastic.

[6] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

TABLE 1.—Effect of the homo-aza-steroidal ester of [*p*-[bis(2-chloroethyl)amino]phenyl]acetic acid on T8 tumor (schedules causing toxic deaths are not included)

Treatment schedule			No. of rats	Mean tumor volume (cm^3)		T/C%		No. of cures	Mean survival of noncured rats (days)
Dose and route (mg/kg)		Day of treatment		Day 10	Day 20	Day 10	Day 20		
Controls			9	1.50	15.00				46
Homo-aza-steroidal ester									
Ip	10	11–18	3		0.40		2.7	1	54
	10	2–9	5	0		0		3	62
	25	10,15	8		0.66		4.4	4	75
	25	2,6	8	0		0		8	
Sc	10	2–9	5	0		0		—	55
	25	2,6	4	0		0		2	64
	50	10,15	3		0		0	3	
Oral	10	11–18	6		1.13		7.5	2	69
	25	10,15	7		0.25		1.7	3	78
	50	2	3	0		0		3	
Cyclophosphamide									
Ip	10	2–9	5	0		0		5	
	25	10,15	6		0.95		6.3	—	70
	25	2,6	3	0		0		3	
	50	2	3	0		0		3	
Homo-aza-steroid									
Ip	25	2,6	4	0		0		1	56
Sc	50	2	4	0.45		30		—	52

TABLE 2.—Effect of the homo-aza-steroidal ester on B16 melanoma (schedules causing toxic deaths are not included)

Treatment schedule					
Dose and route (mg/kg)	Day of treatment	No. of mice	Mean tumor volume (cm^3)	T/C%	Mean survival (days)
Controls		10	1.40 (Day 17)		29
Ip 25	10,15	4	0.95 (Day 17)	68	29
50	2	5	0 (Day 10)	0	34
Sc 50	2	4	0.22 (Day 10)	18	45

TABLE 3.—Effect of the homo-aza-steroidal ester on Th-B angiosarcoma (only some representative schedules are included)

Treatment schedule					
Dose and route (mg/kg)	Day of treatment	No. of mice	Mean tumor volume (cm^3, Day 20)	T/C% (Day 20)	Mean survival (days)
Controls		10	2.00		26
Ip 5	2–9	5	0	0	Two cures, 26
Sc 10	11–18	5	0.70	35	80
25	10,15	5	0.50	25	62
10	2–9	3	0	0	Three cures

The toxic effects on various systems and functions, especially on hematopoiesis, in animals receiving therapeutic doses and in animals surviving toxic doses are reversible in 15–20 days. Twenty-five days after the administration of the compound no signs of toxic effects were found.

DISCUSSION

It remains to be determined if this homo-aza-steroidal ester is more active than the other steroidal esters. The hypothesis that the homo-aza-steroidal part of the molecule also has an alkylating effect is still under investigation. It seems possible that the lactam group may be involved in covalent bond formation. However, the homo-aza-steroidal esters of the *p*-phenylacetic nitrogen mustard are very interesting with regard to their cytostatic action and we believe it is worthwhile to further extend the testing of these compounds.

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ANNOUNCEMENTS

American College of Physicians: Course on Advances in Clinical Cancer

Advances in Clinical Cancer will be held on November 12–15, 1973, at the Westbury Hotel, 480 Sutter Street at Powell, San Francisco, California. This postgraduate course has been designed, in cooperation with Continuing Education in Health Sciences, University of California, San Francisco, to review information concerning advances in the diagnosis and management of certain malignant diseases as encountered in clinical practice by the general practitioner as well as the specialist. It will be presented by authorities in the field of diagnosis and treatment in a variety of oncologic problems in the non-hematologic malignancies (ie, solid tumors). The problems encountered in major common malignancies are approached in multidisciplinary methods. During the morning sessions, fundamentals are presented. In the afternoon sessions, workshops, tumor boards, and panels are scheduled during which registrants are encouraged to participate actively in the programs.

A block of rooms has been reserved for this conference at the Westbury Hotel. Please make your reservations directly with the hotel. Be sure to identify yourself as being with the conference.

This program is acceptable for 25 hours of Category I credit towards the certificate in Continuing Education for the California Medical Association and the American Medical Association.

For further information write to Edwin M. Jacobs, MD, Program Director, University of California, San Francisco, Calif 94122.

Traineeships in Clinical Oncology

University of Rochester Medical Center, Rochester, New York

Four Traineeships in Clinical Oncology are offered annually at the University of Rochester Medical Center under the supervision of the interdisciplinary Clinical Cancer Training Committee. Coordination of the program is facilitated by an interdepartmental Division of Oncology which brings together clinicians and basic scientists with primary interests in neoplastic disease. The Program is designed to provide broad experience in diagnosis, management, and investigation of neoplastic diseases. Intensive experience is available in chemotherapy, radiotherapy, surgical oncology, gynecologic oncology, endocrinology, hematology, pediatric oncology, and surgical pathology through a flexible program individualized for each trainee depending on past experience and future plans.

Applicants with primary training in medicine, surgery, gynecology, pediatrics, or radiation therapy are eligible. Two-year appointments are encouraged, providing opportunity for both clinical training and investigative experience, including laboratory investigation in tumor biochemistry and tumor immunology.

Traineeships ordinarily begin July 1. Applications should be submitted by November 15. Applicants must be citizens of the United States or non-citizens with permanent visas.

Inquiries for additional information and application forms should be sent to: Richard F. Bakemeier, MD, Chairman, Clinical Cancer Training Committee, University of Rochester School of Medicine and Dentistry, 260 Crittenden Blvd, Rochester, NY 14642.

Special Announcement

ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC.

The scientific sessions of the 65th Annual Meeting of the American Association for Cancer Research, Inc., will be held at The Rice Hotel, Houston, Texas, from 8:30 A. M., Thursday, March 28, until 5:00 P. M., Saturday, March 30, 1974.

Registration will be conducted only during the afternoon and evening of March 27 and during the course of the meeting. Nonmembers may obtain programs and purchase abstracts of the papers at the time of registration. By action of the Board of Directors, a registration fee of $15.00 will be charged each member and nonmember attending the scientific sessions; a special rate of $5.00 will be charged medical and graduate students who present a statement, signed by the registrar, dean, or department head of their university or college, confirming their status as students. Admission to the scientific sessions will be restricted to properly registered persons.

A copy of the detailed regulations involving submission of abstracts and presentation of papers will be sent to each active member of the Association as part of the customary Annual Letter to be issued in October 1973. Corresponding members and nonmembers may obtain copies of the regulations and hotel reservation cards from the Secretary-Treasurer. The deadline for receipt of abstracts is December 1, 1973. Abstracts that are submitted in conformity with the regulations and considered of acceptable quality will be published in the March 1974 PROCEEDINGS of the American Association for Cancer Research, which will be mailed by the publisher to all paid-up subscribers to the journal CANCER RESEARCH.

Hugh J. Creech, Secretary-Treasurer
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